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PROJECT ADMINISTRATION DATA SHEET

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Project No. G-33-Q05 (Continuation of G-33-Q04) ~~GTRI~~/GIT DATE 3 / 30 / 84

Project Director: Dr. Leon H. Zalkow School/~~Dept~~ Chemistry

Sponsor: DHHS/PHS/NIH/National Eye Institute

Type Agreement: Grant No. 5 R01 EY03342-05

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Title: Antiglaucoma Compounds from Cannabis Sativa

ADMINISTRATIVE DATA

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See Attached NIH Supplemental Information Sheet for Additional Requirements.

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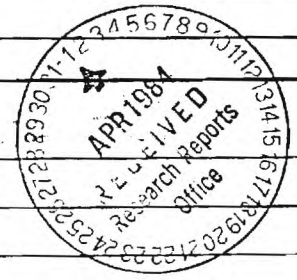

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Date 3-18-87

Project No. G-33-Q05 School/~~Lab~~ XXX Chemistry

Includes Subproject No.(s) N/A

Project Director(s) Leon H. Zalkow ~~GTRC~~ XXXX / GIT

Sponsor DHHS/PHS/NIH/National Eye Institute

Title Antiglaucoma Compounds from Cannabis Sativa

Effective Completion Date: 8/31/85 (Performance) 11/30/85 (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
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Isolation of Ocular Hypotensive Agents From Cannabis sativa

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Abstract: Recent work in our laboratories has shown that a hydrophilic fraction from *Cannabis sativa* (marihuana) has extremely potent intraocular pressure (IOP)-lowering activity as measured in albino rabbits when delivered by intravenous injection. A crude extract reduced IOP by 50-60 per cent (to the episcleral venous pressure) at dosage levels of about 500 μg/animal. Fractionation of this material by solvent extraction, high-performance liquid chromatography, and gel filtration chromatography has produced samples with high activity at 50 μg/animal. The active material has been shown to be noncannabinoid and of high molecular weight.

CANNABIS SATIVA (marihuana) has had an interesting history as a medicinal plant and intoxicant for at least the last several thousand years. The chemistry of this plant has been intensively studied for many years, and a recent review identified some 420 natural products that have been isolated.[1] The medical use of marihuana in the United States ended about 1937. In the early 1960s, the rapid expansion of the illegal use of *cannabis* encouraged new investigations into the effects of marihuana consumption. It was not until 1964 that it was shown that the main psychoactive compount in *cannabis* was Δ^9-tetrahydrocannabinol (THC).[2]

In terms of the ocular response to *cannabis*, it had been known for many years that smoking or oral consumption causes conjuctival hyperemia.[3] However, it was not until 1971 that Hepler and Frank[4] reported a lowering of intraocular pressure (IOP). They found that when marihuana cigarettes containing 18 mg THC were smoked, the IOP of 13 normotensive volunteers changed by +4 to −45 per cent, averaging about −25 per cent. These findings were confirmed in other studies with both normotensive and hypertensive volunteers[3,5,6] and in extensive animal studies, primarily by Green and co-workers.[3]

In summary, it can be stated that marihuana, THC, and several other related compounds have good activity in lowering IOP and thus have the potential to be useful in the treatment of glaucoma. However, as of this time, an effective, nonpsychoactive, and easy to deliver cannabinoid drug has yet to be developed and to be shown to lower IOP and save vision.

In 1978, an unusual report appeared[7] indicating that a nonpsychoactive preparation had been made from marihuana that was useful in the treatment of glaucoma. Since the details of the experimental work were not included, it was not possible to determine how these "Cannabis Eye Drops" were prepared or tested, either in glaucomatous volunteers or in test animals. For a number of reasons we assumed that the solvent used to prepare this "nonpsychoactive" material must have been water. However, we were unable to demonstrate any topical activity in rabbits using water ex-

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tracts. On the other hand, intravenous injections of the crude aqueous extract of *Cannabis sativa* showed very strong IOP-lowering activity in rabbits, and such an extract forms the basis for the work reported herein.[8]

Methods

Samples of *Cannabis sativa* (Mexican) were obtained from the University of Mississippi Research Institute of Pharmaceutical Sciences through the auspices of the National Institute of Drug Abuse (NIDA). The plant material was ground and extracted with water. The water extract was filtered and then freeze dried. Liquid chromatographic separations were performed on either a Water's Prep 500 system or a Laboratory Data Control System using C-18 reverse-phase columns. Continuous extractions of solid extract were done in a Soxhlet apparatus. Samples were dialyzed against distilled water using cellulose tubing with a 12,000 molecular weight cutoff (Fisher). Gel filtration chromatography was performed with Pharmacia Sephadex G-25 resin in a column 20 × 2.5 cm using distilled water as eluant and ultraviolet detection at 254 nm. Ion exchange chromatography was done on DEAE cellulose (Whatman D-52) using 0.05 *M* Tris buffer (Sigma) adjusted to pH 6.7 with hydrochloric acid; the ionic strength varied with sodium chloride. A column 35 × 2.5 cm was used with the above solvents, and detection was at 254 nm. Carbohydrate analyses were done by the phenol-sulfuric acid method,[9] whereas protein was analyzed by the method of Lowry.[10]

IOP screening was performed in adult albino rabbits (two to six were used for each test) weighing 2.5 to 3.5 kg each, of either sex. Before the initial application of a topical drop or intravenous injection, a baseline IOP was obtained; two readings of IOP were made 30 minutes apart using an Alcon Pneumotonograph calibrated for the rabbit eye. After delivery of the drug, readings were taken hourly up to 7 hours and then usually at 24 hours. In some experiments, other time periods were employed. Rabbits were preconditioned to the laboratory regime prior to the drug testing procedure. Error analysis for IOP readings at each time period showed that with two rabbits (four eyes), the standard error was usually 5 to 12 per cent change in IOP; with six rabbits (12 eyes), the standard error was usually 2 to 6 per cent change in IOP.

Results

The water extract contained approximately 2.7 Gm dissolved solids per 100 ml, and the total solids represented about 22 per cent by weight of the air-dried plant. Topical application of the water extract on rabbit eyes resulted in no significant change in IOP. Consequently, intravenous injection was tried. Activity was observed at 1.0 and 0.1 ml per animal (Fig. 1). As will be detailed later, the pattern of change involv-

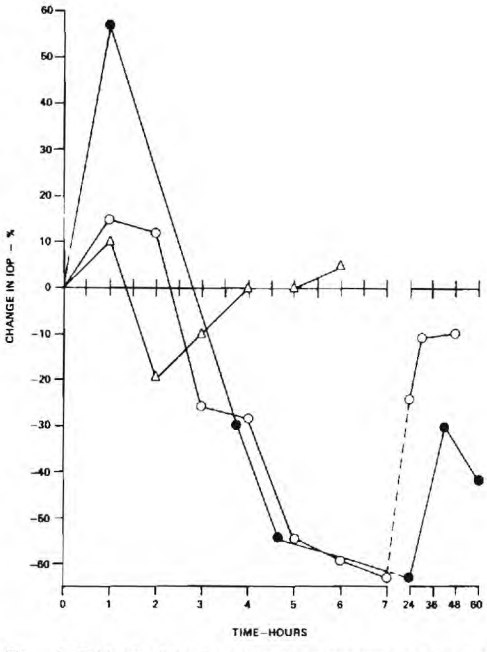

Fig. 1. Effect of intravenous aqueous extract of Cannabis sativa on rabbit IOP: (•) 1.0 ml (~ 25 mg solids); (o) 0.1 ml (~ 2.5 mg solids); (Δ) 0.01 ml (~ 0.25 mg solids);

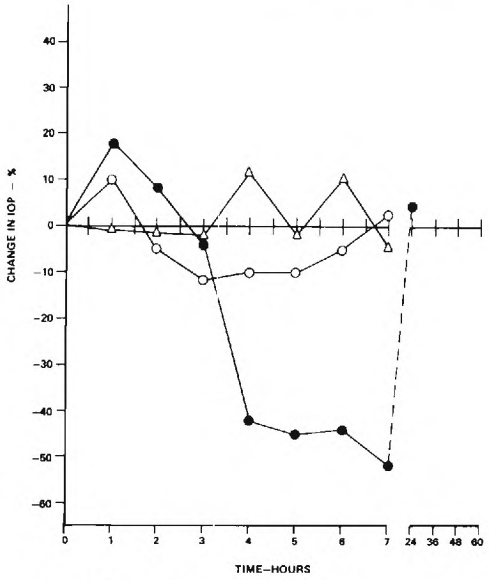

Fig. 2. IOP testing of fractions from reverse-phase chromatography. Intravenous injection in rabbits at 0.5 mg per animal: (Δ) fraction 1—most polar; (•) fraction 2--less polar; (o) fraction 3—least polar.

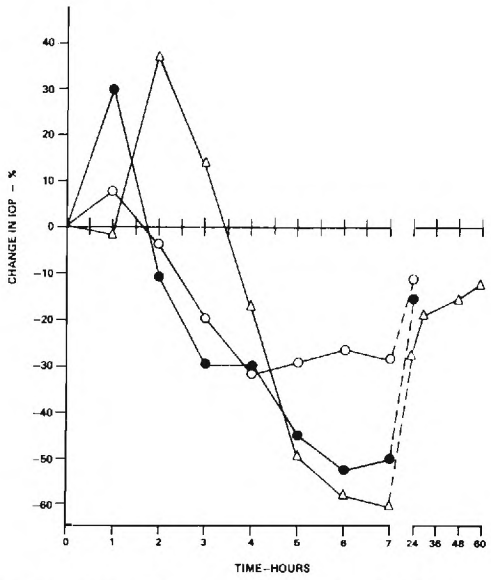

Fig. 3. IOP testing of material from Soxhlet extraction of freeze-dried extract. Material soluble in water, but not soluble in n-butanol, acetone, or methanol: (Δ) 0.50 mg per animal; (•) 0.25 mg per animal; (o) 0.10 mg per animal;

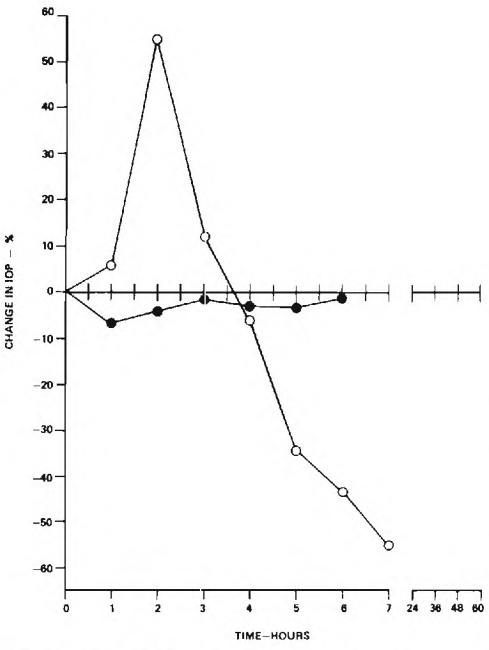

Fig. 4. IOP testing of dialysis fractions. Intravenous injection in rabbits at 0.20 mg per animal: (o) retentate; (•) dialyzate.

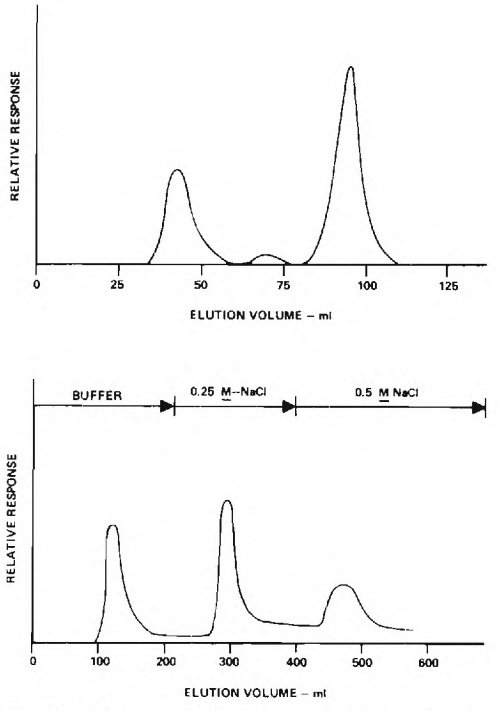

Fig. 5. Chromatography of aqueous extract of Cannabis sativa. Top, Sephadex G-25 gel filtration; bottom, DEAE cellulose ion exchange.

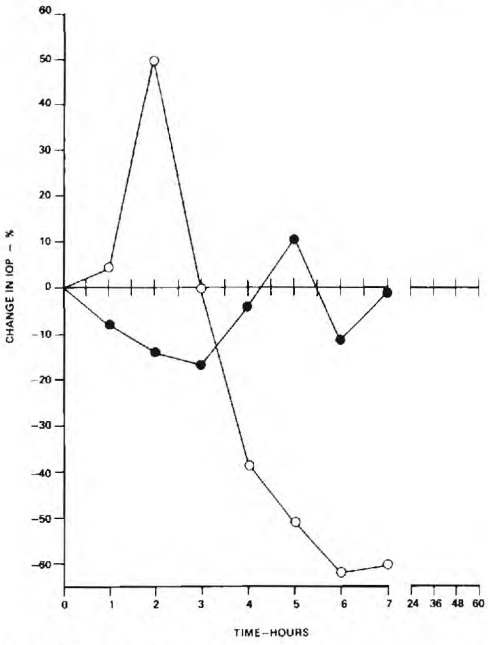

Fig. 6. IOP testing of fractions from gel filtration chromatography on Sephadex G-25. Intravenous injection in rabbits at 0.20 mg per animal: (o) void volume material; (•) later eluting material.

ing an initial ocular hypertensive phase followed by the rapid onset of a very powerful hypotensive effect is quite typical.

Initial attempts to fractionate the extract centered on liquid chromatography. High-performance liquid chromatography (HPLC) using a low loaded C-18 reverse-phase column and water as the eluting solvent indicated that some separation of material was possible under analytical conditions using UV detection at 254 nm. Therefore, a large-scale separation was made using the Water's Prep 500 equipped with a similar column. The refractive index detector did not reveal any clear separation; a large number of fractions were taken and each analyzed for IOP-lowering activity. Thus, 10.0 Gm freeze-dried extract was stirred with 100 ml water for 1 hour and filtered, leaving about 3 Gm insoluble dark material. The solution was injected on the column, and after elution of the void volume, fractions were taken. The first 500 ml contained 4.51 Gm (64 per cent) inactive material. The next 500 ml contained 0.56 Gm (8 per cent) active material. The balance of the eluted material (solvent 100% water to 100% methanol) was 1.21 Gm (17 per cent) and was inactive. The results of IOP screening of these fractions are shown in Fig. 2.

Although the above procedure did fractionate some active material, other methods of purification were sought. One simple procedure was solvent extraction of the freeze-dried solid with various organic solvents. When 18.33 Gm freeze-dried extract was extracted successively with *n*-butanol, acetone, and methanol, fractions were obtained that contained 0.68 Gm (3.7 per cent), 0.51 Gm (2.8 per cent), and 6.07 Gm (33.1 per cent), respectively. None of these was active. The residue (ca 11 Gm) was washed with a large volume of hot water, and the clear, dark solution was freeze dried. This yielded 7.4 Gm (40 per cent) material that was quite active. Figure 3 summarizes IOP screening of this fraction.

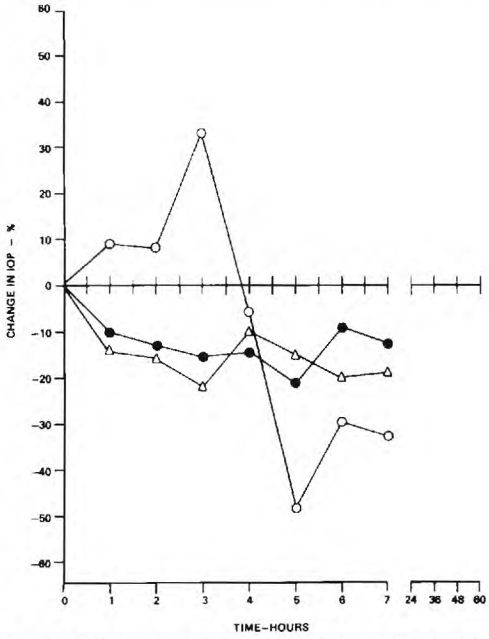

Fig. 7. IOP testing of fractions from DEAE cellulose ion exchange chromatography. Intravenous injection in rabbits at 0.20 mg per animal: (o) fraction 1 (buffer only); (•) fraction 2 (buffer plus 0.25M NaCl); (Δ) fraction 3 (buffer plus 0.50M NaCl).

Since the active material seemed to be totally insoluble even in polar organic solvents but readily soluble in water, the possibility of the active material being polymeric in nature was investigated by dialysis. In one experiment, 112.8 mg was dialyzed twice for 24 hours each. The retentate and dialyzate were freeze-dried to yield 22.4 mg (19.9 per cent) and 88.6 mg (78.5 per cent), respectively. Screening of these material is shown in Fig. 4 which shows the high activity of the retained material versus the dialyzate.

Similar results were obtained on gel filtration chromatography, as shown in part of Fig. 5. When 60.0 mg material was chromatographed using water as the eluting solvent, the first material eluted off the column (void volume) gave 10.2 mg, with the later eluting material yielding 46.6 mg. IOP screening of these fractions (Fig. 6) indicated that the activity was in the void volume material.

Another separation was achieved when 199 mg extract was chromatographed on the ion exchange resin DE-52 (DEAE cellulose). In this experiment the eluting solvent, in all cases, was 0.05*M* Tris buffer at pH 6.7. After the first fraction was eluted (15.3 mg), the ionic strength was changed to include 0.25*M* NaCl, and this gave a second fraction (10.8 mg); a third fraction was obtained at 0.50*M* NaCl (10.4 mg). This chromatography is shown as part of Fig. 5. All fractions were dialyzed to remove buffer and salt and therefore contain no low-molecular-weight material. The IOP screening results for these fractions are summarized in Fig. 7.

The first fraction eluted off the G-25 column (void volume fraction) was analyzed for carbohydrate using *D*-glucose and *D*-galactose (1:1.5) as a standard. Duplicate analyses gave 33.4 and 36.5% carbohydrate. This fraction also gave a strong positive color reaction for protein.

Discussion

The aqueous extract of *Cannabis sativa* obviously contains compound(s) with extremely powerful IOP-lowering activity when delivered intravenously to rabbits. The crude extract shows this activity at about 2.7 mg per animal but not at 0.27 mg per animal. Characteristically, there is a rise in IOP at 1 to 2 hours after injection and then a very rapid fall in IOP at 3 to 4 hours. With sufficient dose, the IOP falls to about 60 per cent below baseline, which approaches the maximum fall possible since the absolute IOP is reduced to the normal episcleral venous pressure. None of the other drugs commonly used in glaucoma therapy has such a dramatic effect.

Liquid chromatography was used to try to fractionate these crude extracts. Although separation of active fractions was achieved, the expected increase in activity was not observed. A simpler method of purification, namely, extraction of the freeze-dried solid first with organic solvents and then with water, produced material that was very active at 100 μg per animal (and in some experiments at even lower dosages).

The chemical nature of these active compound(s) was revealed from dialysis experiments. The retentate, which represented about 20 per cent of the crude fraction, had very high activity at 200 μg per animal, whereas the dialyzate was inactive at the same dosage. It would thus appear that the active material was a high-molecular-weight polymer. This was confirmed by gel filtration chromatography on Sephadex G-25; the void volume eluting material was active at 200 μg per animal, whereas the later eluting material was inactive.

Some information as to the ionic nature of the active compound(s) was obtained by chromatography on DEAE cellulose. The least acidic material, which was eluted with 0.05*M* Tris buffer at pH 6.7, had the highest IOP-lowering activity. More acidic fractions that eluted at higher ionic strength

showed weaker but real activity (confirmed at higher dosages).

The active material, eluting at the void volume in gel filtration chromatography, was quantitatively analyzed for carbohydrate using the phenol-sulfuric acid method. This showed that the material contained about 35 per cent carbohydrate. Protein analysis has shown a strong positive test by the Lowry method.

These results are consistent with those of Wold et al.[11-13] who have found and partially characterized a glycoprotein from aqeuous extracts of South African and Thai *Cannabis sativa*.

Summary

The aqueous extract of *Cannabis sativa* (Mexican) has been shown to contain compound(s) with very powerful intraocular pressure (IOP)-lowering activity. This screening was done by intravenous injecton in albino rabbits. Topical application of this extract did not lower IOP. The aqueous extract has been fractionated in a number of ways. It has been shown that the active component(s) are of high molecular weight as indicated by dialysis experiments and by gel filtration chromatography. Ion exchange chromatography has given indications that there may be more than one active component; the least acidic of these appears to have the greatest activity. Preliminary analysis of the high-molecular-weight fraction from gel chromatography has shown that it contains a considerable amount of carbohydrate and protein.

Acknowledgments

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Current Eye Research

Ocular and systemic responses to water soluble material derived from Cannabis sativa (marihuana)

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ABSTRACT

A water soluble material, isolated from Cannabis sativa, has been tested in albino and pigmented rabbits and rhesus monkeys for both ocular and systemic effects. Intravenous administration produced a dose-related fall in intraocular pressure in both albino and pigmented rabbits with concentrations as low as 0.005 mg/animal being effective, but no response was found in monkeys. High concentrations (0.2 to 1 mg/animal) induced a hypertensive phase in intraocular pressure prior to the ocular hypotension; higher concentrations (2 or 5 mg/animal) also induced antidiuresis and general relaxation. Tachyphylaxis was found to repeated daily injections. Alpha and ß-adrenergic antagonists caused some reduction of the hypertensive phase but had no effect on the hypotensive phase. Superior cervical ganglionectomy did not influence the time course of the intraocular pressure response. Indomethacin inhibited the hypertensive intraocular pressure phase but was ineffective against the hypotensive phase. Systemic blood pressure was unchanged following intravenous administration of 0.2 mg material/animal. Aqueous humor protein concentration was increased at both 1 and 6 hours after intravenous administration, becoming greater at the later time. Aqueous humor turnover rate was substantially reduced reaching a minimum 8.75 hours after administration. Topical administration was ineffective in eyes when the epithelium was removed in rabbits with and without pretreatment with aspirin. Neither gastric nor suppository administration of large quantities (10 mg or greater) of material had any influence on intraocular pressure.

INTRODUCTION

The chemical constituents of marihuana, Cannabis sativa, L, are well-known (1), and most of the pharmacological studies on marihuana extracts have been directed towards comprehension of the effects of cannabinoids. The cannabinoids have been identified as evoking the majority of the pharmacological effects of marihuana and are associated with the well-recognized euphoric or psychoactive effects of the parent material (2,3). The ocular effects of marihuana and the cannabinoids have been documented in both man and animals (4).

The cannabinoids are exclusively soluble in organic solvents, but also present in the plant material are water soluble compounds. Some of the physiological and pharmacological activities of certain of these compounds have been indicated previously. These activities include atropinic and muscarinic-like action (5), as well as aldose reductase inhibition (6).

An extract of Cannabis, for which the solvent was not specified, was described as acutely reducing the intraocular pressure (IOP) of dogs. When used in man as topical drops, a progressive fall in IOP occurred over a period of about 6 weeks (7). This study indicated that other biologically active compounds could be extracted from Cannabis which caused ocular changes and our studies were directed towards comprehension of the effect of water soluble compounds on IOP and their mode of action.

MATERIALS AND METHODS

Drugs and preparation

Cannabis sativa L, Mexican variety, was supplied by the University of Mississippi, Research Institute of Pharmaceutical Sciences. Water extracts were prepared as described previously (8) and various materials, prepared by various chromatographic techniques, were screened for IOP reducing activity using an initial intravenous test dose of 0.2 mg/animal.

Following the initial identification of a specifically active material which caused a marked fall in IOP, this drug was chosen to investigate the pharmacological activity. The material of choice (marihuana - derived material or MDM) was

that remaining after Soxhlet extraction of freeze dried crude water extract which was insoluble in butyl alcohol, acetone or methanol but which was water soluble (8). This material was by far the most potent of those extracted, markedly reducing IOP when given intravenously, with its extraction from the crude material being duplicated with ease.

The MDM was readily soluble in water and each solution was made up fresh in 0.9% saline. Irrespective of the amount of MDM administered per animal the volume was 1 ml for intravenous injection.

Animal testing

Rabbits. Both albino and pigmented rabbits (2-3 kg) were used.

a) IOP determinations. Animals were restrained in canvas bags tied at the neck and the IOP measured using a calibrated Alcon Pneumatonograph. Corneas were anesthetized with one drop of tetracaine (proparacaine hydrochloride) which was washed off after 5 to 10 seconds with at least 1 ml of 0.9% saline. Measurements were made (relative to the time of drug administration) at -40 min., -30 min., 0, and at each successive hour up to 7 hours on each test day. Frequently a 24 hour hour reading was made. The -40 min. reading was discarded and the -30 and 0 values were averaged to provide a mean time-zero value for each rabbit. Percentage changes in IOP were calculated relative to the time-zero prior to statistical analysis. Control series were frequently performed to determine any non-drug related changes occurring with time. MDM was also boiled for 30 minutes and administered intravenously at 1 mg/animal.

b) Antagonists. Several drugs were used as potential antagonists; α-adrenergic blocking agents, phenoxybenzamine and phentolamine or β-adrenergic blocking agents, sotalol and propranolol were used at 0.5 or 5 mg/kg and were all given intravenously. Timolol (Timoptic), 0.5%, was unilaterally applied topically 1 hour prior to intravenous MDM and every hour thereafter up to 8 hours. Indomethacin (20 mg/kg) was given intraperitoneally. Hexamethonium chloride and tripelennamine, an antihistamine, were both given intravenously at 10 mg/kg and 2 or 4 mg/kg, respectively. The adrenergic antagonists, as well as indomethacin and tripelennamine were all given at 0 time with MDM given 1 hour later. IOP changes given are relative to the intravenous administration of MDM. Hexamethonium chloride was given 6 hours after the intravenous MDM.

Animals were anesthetized with xylazine and ketamine for the unilateral surgical removal of a superior cervical ganglion. At 10 days post surgery, eyes were tested for adrenergic supersensitivity with 0.1% norepinephrine administered to both eyes. Successful surgery was adjudged by mydriasis in the eye of the operated side. Animals were used at least 3 days following testing for supersensitivity.

c) Topical administration. MDM was dissolved in 0.9% saline at various concentrations from 1 to 10% and applied unilaterally in a topical 50 µl drop in the presence and absence of benzalkonium chloride or cetylpyridinium chloride. In some experiments the corneal epithelium of both eyes was removed under topical anesthesia with a Gill corneal knife, with saline subsequently administered in one eye and MDM in the contralateral eye. In the latter experiments an aspirin suppository was given to the rabbit 1 hour prior to topical application of MDM.

d) Other routes of administration. Oral administration was performed via a gastric tube inserted into the stomach through the esophagus. Suppository administration was achieved using gelatin suppositories which were halved and MDM placed between the two halves; finger heat was sufficient to reseal the suppository. Intravitreal injections were made in a 10 µl volume via a Hamilton syringe and a 30 guage needle into the center of the vitreous as observed through the cornea. The site of injection was about 6 mm posterior to the limbus. Administration of MDM at concentrations of 5, 10 or 25 µg/0.1ml into the 3rd ventricle of rabbits was made as described previously (9,10): IOP was measured using a floating-tip applanation tonometer and body temperature measured with a rectal probe.

e) Blood pressure. Systemic blood pressure

was measured by direct femoral artery cannulation, using a Sanborn transducer connected to a recorder. Blood pressure was measured either continuously over 8 hours under urethane anesthesia, or between 5 and 8 hours after administration of MDM. In the latter experiments the animal was conscious for the first 5 hours and was subsequently anesthetized and cannulated.

f) Aqueous humor flow rate. 1) Fluorometry: Aqueous humor flow rate was determined using fluorophotometry (11), using an apparatus similar to that described by Waltman and Kaufman (12). Fluorescein was administered topically to the cornea and the aqueous humor fluorescein concentration was determined at intervals (13) both before and after administration of 0.5 mg per animal of MDM.

ii) Inulin dilution: Measurements of aqueous humor formation rate and pseudofacility were made 4 to 6 hours after intravenous injection of MDM in the urethane anesthetized rabbit using an inulin dilution technique (14). The technique consists of perfusion of inulin through an eye in which the IOP is held constant at the drug-induced pressure and the dilution of inulin is monitored, thereby allowing calculations of aqueous formation rate. Pseudofacility was determined in a similar manner but with the IOP raised by 5 mmHg above the drug-induced value.

g) Aqueous humor protein concentration. One hundred µl of aqueous humor was removed from the eyes of rabbits at either 1 or 6 hours after administration of MDM. Protein determinations were made using the Lowry technique (15).

Rhesus monkeys. Conscious rhesus monkeys (3 kg) were placed into restraining chairs specifically designed to allow measurements of IOP was measured at 0, 1, 3 and 6 hours with drug administration made after the 1 hour reading. The chair is designed to tilt and hold the monkeys in a supine position for tonography. Control series were run at various times during the entire study using the same monkeys. A calibrated Alcon Pneumotonograph was used for IOP and total outflow facility measurements (tonography was performed with a 10 g weight); the latter were performed immediately following the IOP measurements. MDM was either given intravenously, or topically in eyes in which the cornea had been partially scraped. At the end of all procedures neosporin ointment was prophylactically instilled onto the cornea to prevent infection.

RESULTS

Rabbits

a) IOP determination. Since preliminary data (8) indicated that the crude water extract was inactive when applied topically to rabbit eyes, but caused a marked fall in IOP when given intravenously, all tests reported in this section were performed with intravenous injection.

A dose related fall in both albino and pigmented rabbit IOP was found following intravenous injection of the Soxhlet extracted MDM (Table 1). Concentrations as low as 0.005 mg/animal (approx. 0.002 mg/kg) were effective in significantly reducing IOP. Characteristic of high concentrations, but not revealed by the statistical analysis in Table 1, is an increase in IOP at 1 or 2 hours after injection. Since the hypertensive phase occurs at different times in different individual animals this phase is not always noted in a group analysis (Table 1), although in some cases (see Tables 2 and 3) the hypertensive phase is revealed. At even higher doses several reactions were seen: the animals became listless and completely flaccid at either 2 or 5 mg/animal, which with the latter dose lasted for up to 3 or 4 days (with a lower IOP for about 2/3rds of this time), and the urine output of the animals was significantly decreased.

Repeated daily injections of 0.5 mg/animal of MDM showed the characteristic IOP fall in IOP on day 1; on the next 4 successive days no IOP change was seen. Thus, a tachyphylaxis was found to repeated MDM administration. This was also found when 0.01 mg/animal was given on day 1 and 0.1 mg/animal was given in successive days.

Previously boiled MDM failed to induce a significant fall in IOP, indicating its sensitivity to temperature (Table 1).

Table 1: Effect of a water soluble marihuana-derived compound on rabbit IOP.

Dose (mg/animal)	n	Time (hours)								
		0	1	2	3	4	5	6	7	24
1	6	20.4^{+} ±0.5	22.4 ±1.7	17.4 ±1.6	19.9 ±1.9	13.3* ±1.1	9.6* ±0.6	7.2* ±0.6	7.4* ±0.5	17.8 ±0.7
			+17.8‡ ±9.7	-12.3 ±8.8	+0.6 ±11.2	-33.8* ±5.8	-51.7* ±4.0	-64.2* ±3.2	-62.7* ±2.9	-11.9* ±3.0
0.1	6	19.8^{+} ±0.2	+ 2.1 ±10.4	-18.4* ±4.5	-30.5* +6.0	-41.8* ±3.1	-40.4* ±3.3	-47.3* ±3.6	-48.0* ±4.0	-
0.01	6	23.4^{+} ±0.4	-12.9‡ ±2.4	+6.2 ±8.3	-14.6 ±7.6	-40.4* ±5.8	-48.3* ±4.6	-58.8* ±4.1	-65.8* ±1.0	-5.3 ±7.2
0.005	5	24.6^{+} ±1.0	-7.4‡ ±3.8	-22.1* ±4.0	-28.6* ±5.5	-22.5* ±6.8	-24.6* ±5.8	-21.4* ±1.8	-26.7* ±5.2	-6.2 ±3.5
0.001	6	21.6^{+} ±0.4	-2.6‡ ±2.1	-6.3 ±1.6	-5.8 ±2.6	-7.5 ±2.1	-3.7 ±2.7	-10.8 ±3.5	-10.7 ±3.6	-
0.0001	6	21.5^{+} ±0.6	-14.2‡ ±3.6	-8.3 ±1.4	-1.1 ±1.5	-3.4 ±3.3	±2.0 ±3.4	+0.1 ±1.5	+1.8 ±1.2	+6.9 ±1.9
1 (boiled)	6	20.4^{+} ±0.6	-4.2 ±2.4	-8.4 ±2.3	-7.9 ±2.7	-11.7 ±2.3	-12.7 ±3.5	-12.0 ±4.0	-	-

Values are the mean ± SEM, n, number of animals. $^{+}$, absolute values of IOP in mmHg, ‡ values given as percentage of original IOP; -, indicates fall, +, indicates increase in IOP. All 0 time values are mean IOP ± SEM. The difference between the decrease in absolute IOP and the percentage IOP is that each percentage fall represents the mean calculated for each eye and its base line value, whereas the absolute IOP's shown are the mean of those absolute values. *, significantly different from base-line IOP, $p < 0.05$.

b) Antagonists. In the determination of interaction with adrenergic blocking agents, a standard dose of 1 mg or 0.1 mg per animal of MDM was used (Table 2). Phenoxybenzamine, 0.5 mg/kg, had no effect on either the hyper- or hypotensive effect of MDM but 5 mg/kg suppressed the hypertensive effect (Table 2). Propranolol, at the same concentrations, caused similar effects. Sotalol at either 5 mg/kg, which reduced IOP by about 15-18% alone, or 0.5 mg/kg which alone reduced IOP by 10%, had no effect on the hyper- and hypotensive drug effects. Phentolamine also had no effect on the IOP change induced by MDM (Table 2). Timolol at 0.5% given topically at every hour did not block the IOP-lowering effects of MDM. Indomethacin blocked the hypertensive IOP phase induced by MDM but had no effect on the hypotensive phase (Table 3). Hexamethonium chloride, injected at 6 hours after the administration of MDM, had no effect on the hypotensive IOP phase. Pretreatment with tripelennamine also had no effect on the IOP pattern caused by MDM (Table 3). Unilaterally ganglionectomized rabbits showed a similar pattern of IOP change in both eyes following intravenous administration of 0.2 mg/animal of MDM (Table 3).

c) Topical administration. Topical administration of MDM on an intact cornea was ineffective is causing a change in IOP. Various concentrations were used (up to 10%) in the presence and absence of benzalkonium chloride (0.04%) and cetylpyridinium chloride (0.02%). Since no effect was found under these conditions and other data indicated that MDM

Table 2: Effect of adrenergic antagonists on the IOP fall induced by marihuana-derived material.

Drug and Concentration	n	Time (hours)							
		1	2	3	4	5	6	7	24
Phenoxybenzamine (0.5 mg/kg)	3	-6.5 ±3.8	+35.7* ±14.1	+13.7 ±7.3	-24.8* ±6.2	-31.8* ±3.8	-37.3* ±5.0	-49.7* ±1.5	-11.8 ±8.8
Phenoxybenzamine (5 mg/kg)	3	-11.2 ±5.2	-17.7 ±9.2	-13.7 ±6.5	-40.8* ±5.7	-50.0* ±2.0	-54.5* ±3.1	-57.2* ±3.8	-21.5* ±9.9
Phenoxybenzamine alone (5 mg/kg)	2	-11.5 ± 0.5	-23.0* ±2.0	-14.0 ±3.0	-23.0* ±2.0	-18.0* ±2.0	-24.5* ±3.5	-24.5* ±3.5	-3.5 ±0.5
Phentolamine (0.5 mg/kg)	6	-6.0 ±2.2	-5.0 ±4.2	+2.5 ±8.2	-13.6 ±3.9	-43.8* ±2.4	-51.7* ±2.4	-53.6* ±2.6	+2.0 ±3.0
Phentolamine alone (0.5 mg/kg)	4	-14.9* ±2.7	-9.1 ±1.8	-6.6 ±2.7	-10.6 ±3.9	-7.8 ±3.2	-2.4 ±2.1	-0.2 ±2.1	-
Propranolol (0.5 mg/kg)	8	-4.5 ±1.8	+13.8 ±5.9	+5.2 ±5.7	-9.9 ±8.7	-34.9* ±3.7	-45.8* ±3.6	-47.1* ±3.1	-37.7* ±6.0
Propranolol (5 mg/kg)	6	-7.3 ±2.1	-2.6 ±2.6	-7.8 ±8.6	-20.7 ± 6.7	-41.2* ±3.9	-48.6* ±4.8	-53.4* ±4.3	-16.3 ±4.9
Propranolol alone (5 mg/kg)	5	-5.6 ±1.6	-7.5 ±1.8	-6.5 ±2.8	-6.7 ±2.5	-9.3 ±3.9	-6.5 ±3.5	-5.6 ±3.3	-0.8 ±2.2
Propranolol alone (0.5 mg/kg)	5	-4.2 ±3.8	-4.1 ±1.2	-7.4 ±1.0	-6.7 ±3.2	-4.2 ±2.6	-5.2 ±1.5	-3.1 ±2.4	-
Sotalol (0.5 mg/kg)	6	-11.2 ±3.2	+3.4 ±6.1	+3.9 ±6.8	-10.0 ±5.1	-16.2 ±6.1	-45.1* ±2.7	-57.3* ±1.2	-22.9* ±4.6
Sotalol (5 mg/kg)	8	-14.9 ±2.5	+38.8* ± 8.7	+7.6 ±6.3	-14.3 ±3.6	-28.1* ±7.0	-44.3* ±4.7	-47.0* ±4.5	-13.3 ±2.3
Sotalol alone (5 mg/kg)	6	-12.4 ±3.7	-9.3 ±3.8	-13.8 ±2.5	-14.4 ±5.5	-12.7 ±3.4	-17.6* ±4.3	-9.8 ±4.7	-1.3 ±4.2
Timolol (0.5% topical) T^+	5	+0.1 ±1.2	+8.2 ±4.1	+2.7 ±6.0	+8.8 ±4.5	-8.3 ±4.9	-24.9* ±6.6	-35.1* ±8.5	-33.1 ±11.1
UT^+	5	+4.1 ±5.7	+6.0 ±2.6	-0.1 ±12.4	+19.3 ±11.6	-3.2 ±6.7	-19.9* ±8.1	-35.3* ±8.5	-36.9* +10.3
Timolol alone (0.5% topical) T^+	3	-10.9 ±3.9	-5.2 ±2.1	-10.9 ±3.0	-6.0 ±2.4	-11.5 ± 6.1	-9.6 ±3.0	-8.2 ±11.1	-4.9 ±3.7
UT^+	3	-8.6 ±4.9	-7.9 ±7.7	-7.9 ±2.0	+0.2 ±2.2	+9.1 ±9.8	-4.9 ±4.7	-8.8 ±12.4	+0.7 ±0.7

All values are the mean ± SEM of the percentage change in IOP from the base-line readings, established as described in the text. n, number of animals. All drug interactions in this table were made using 1 mg MDM per animal. The initial IOP of each group was about 20 mmHg (see Table 1). *, significantly different from base-line IOP, $p < 0.05$. +, T treated, UT untreated, since drug was applied unilaterally.

Table 3: Effect of various antagonists on the IOP fall induced by marihuana-derived material.

Drug and Concentration	n	Time (hours)							
		1	2	3	4	5	6	7	24
Indomethacin (20 mg/kg)	5	-5.6 ±5.3	-9.8 ±5.6	-18.0* ±3.3	-19.2* ±2.6	-35.3* ±2.8	-46.8* ±3.4	-39.2* ±3.2	-1.3 ±3.3
Indomethacin alone (20 mg/kg)	5	-12.0 ±3.4	-12.3* ±2.6	-5.0 ±2.6	-8.8 ±4.2	-4.6 ±2.5	-7.3 ±4.3	-5.3 ±4.7	-3.1 ±2.7
Tripelennamine‡ (4 mg/animal)	4	+28.2* ±5.6	+19.5 ±8.5	-23.1* ±3.1	-32.6* ±1.9	-40.2* ±4.0	-43.8* ±2.1	-	-
Tripelennamine alone (4 mg/animal)	6	+0.2 ±2.4	+3.7 ±2.5	-0.8 ±2.0	-5.9 ±2.8	-5.4 ±2.8	-7.5 ±2.8	-4.1 ±2.5	+0.8 ±2.8
Tripelennamine‡ (2 mg/animal)	4	+21.5* ±3.4	+45.6* ±13.4	+3.7 ±4.2	-21.0* +1.6	-36.7* ±3.4	-47.1* ±4.7	-	-
Tripelennamine+ (18 mg/animal)	4	-3.8 ±2.4	-2.0 ±4.4	-5.5 ±6.8	-27.1* ±9.5	-29.6* ±8.4	-41.0* ±8.2	-52.7* ±5.1	-35.6* ±2.2
Tripelennamine alone+ (18 mg/animal)	3	-6.2 ±2.6	-9.4 ±2.1	-14.5 ±2.4	-16.3 ±4.2	-9.0 ±2.4	-13.3 ±3.2	-12.7 ±2.3	-3.9 ±1.2
Ganglionectomized eyes	6	+26.9* ±6.5	+13.7 ±15.3	-17.7 ±7.2	-33.1* ±5.7	-48.5* ±3.8	-54.7* ±2.7	-51.3* ±3.2	-21.1* ±4.5
Paired Contralateral eyes	6	-0.2 ±6.2	+3.8 ±6.5	-16.5 ±8.7	-25.5* ±8.6	-43.1* ±5.9	-52.0* ±5.7	-53.6* ±4.6	-19.8* ±3.6
Control, no drug	24	-0.8 ±2.2	-2.9 ±2.2	-3.9 ±2.0	-2.1 ±2.3	-1.1 ±1.9	+0.3 ±1.8	-0.8 ±2.5	-1.3 ±3.0

All values are the mean ± SEM of the percentage change in IOP from the base line readings, established as described in the text. n, number of animals. All drug interactions shown in this Table were made with 1 mg MDM per animal except where indicated by ‡ where only 0.2 mg/animal was used. The initial IOP of each group was about 20 mmHg (cf Table 1). *, significantly different from base-line IOP, $p < 0.05$. All antagonists were given intravenously except + where subcutaneous injections were used.

was of high molecular weight (>12,000 daltons), the epithelium was scraped off in an attempt to allow intraocular penetration. Scraping of the rabbit cornea causes the release of intraocular prostaglandins, thus animals were pretreated with 600 mg suppository aspirin 1 hour prior to corneal epithelial removal and subsequent administration of solutions of various concentrations of MDM. The IOP was unaffected by aspirin alone, and topical applications of MDM were also without effect.

d) Other routes of administration. Intravitreal injection of 1.0 mg of MDM produced a fall in IOP at the 24 hour reading which lasted up to 54 hours (the maximum time followed). At 24 hours the eyes receiving MDM were all inflamed and by 48 hours the corneas were opaque. Lower concentrations (0.1 and 0.01 mg) also caused a large unilateral fall in IOP which was accompanied by a proteinaceous exudate which entered the anterior chamber through the pupil. With these lower concentrations, however, the eye cleared in 3-4 days although the IOP only returned to normal after 5 days. The paired control eyes, which received the same volume of saline as the experimental eyes, showed only a normal change in IOP (ie a fall over 24 hours of about 6%) with no inflammatory reaction.

Neither gastric nor suppository administration of MDM had any effect on IOP. Oral doses as high as 25 mg were used, and, to eliminate any acid effect MDM was held at a pH of 1.2 for 1 hour prior to neutralization and subsequent gastric administration. The latter procedure also produced no IOP effect. A similar acid pretreatment of MDM did, following neutralization, produce an IOP fall indistinquishable from normal after intravenous injection. Suppository administration of 10 mg MDM/animal also induced no effect on IOP. Intraventricular administration of 5, 10 or 25 µg had no effect on IOP or body temperature.

e) Blood pressure. The systemic blood pressure, when measured by either of the two procedures used, was unaffected up to 7 hours after administration of 0.2 mg MDM/animal. The data obtained with constant monitoring of blood pressure, with the rabbit under urethane anesthesia, showed a gradual fall with time which is to be expected with the anesthetic alone(mean blood pressure: treated animals, 0 time, 110 ± 12 mmHg, and at 6 hours, 88 ± 10 mmHg, n = 4; controls, 0 time, 115 ± 9 mmHg and at 6 hours 90 ± 8 mmHg, n = 3). Measurement of blood pressure between 5 and 8 hours after intravenous injection of MDM also revealed no effect (treated animals, 8 hours, 95 ± 10 mmHg, n = 3; controls at 8 hours, 98 ± 8 mmHg, n = 3).

f) Aqueous humor flow rate. i) Fluorophotometry: Aqueous humor flow rates were measured fluorophotometrically following the intravenous administration of 0.5 mg MDM per rabbit. The data are shown in Fig. 1. The aqueous flow rate decreased dramatically from control levels, reaching a minimum at about 8.75 hours; this decrease correlated well with the fall and time course of the IOP change. The maximal fall in flow rate was to about 20% of the original base-line value.

ii) Inulin dilution: The aqueous humor formation rate, measured 6 hours after intravenous administration of 0.2 mg MDM/animal, when the IOP was 8.5 ± 0.8 mmHg, was 1.8 µl/min (n=6) approximately a 40% reduction from the control values of 2.8 µl/min (14). Pseudofacility was

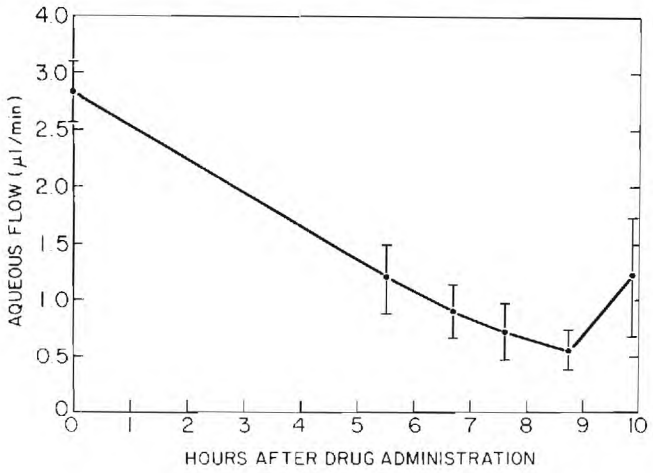

Fig. 1 Aqueous humor flow rate as a function of time after intravenous administration of marihuana-derived material.

Values are the mean ± SD of 7 rabbits.

0.04 ± 0.01 µl/min/mmHg (n=6) which was unchanged from the control value of 0.06 ± 0.01 µl/min/mmHg.

g) Aqueous humor protein concentration. The protein content of aqueous humor was measured in animals receiving 1.0 mg MDM and the results are shown in Table 4. It is evident that the protein concentration is increased during the initial hypertensive phase and continues to increase during the hypotensive phase.

Rhesus monkeys. Intravenous injections of MDM in rhesus monkeys revealed no change in IOP even at concentrations as high as 20 mg per animal (approximately 6 or 7 mg/kg). Initial experiments were made with low concentrations (0.2 mg/animal) and MDM was injected via a syringe with a Millipore cellulose filter attached. Following the failure to induce an effect at 0.2 mg/monkey, corresponding injections were made in rabbits to ensure that the MDM was still active after passage through the filter. A solution was made at a concentration of 0.2 mg/ml and one series of 4 rabbits was injected with unfiltered MDM while another 4 rabbits received injections with a Millipore filter on the syringe. A 50% decrease in activity was found between filtered versus unfiltered MDM. Unfiltered MDM was then used in monkeys (made in sterile

Table 4: Protein concentration of aqueous humor after intravenous injection of marihuana-derived material.

Time (hours)	Protein concentration (mg %)	IOP (% change)	n
0	92	-	2
1	681 ± 73*	+34.5 ± 10.2*	4
6	1245 ± 147*	-46.5 ± 13.9*	4

n, number of eyes; *, significantly different from zero time values.

saline) but still revealed no effect on IOP or behavior.

Topical administration into monkeys eyes which had been partially denuded of corneal epithelium also revealed no change in IOP either in the presence or absence of benzalkonium chloride. The latter was included in an attempt to enhance the transcorneal penetration of the large molecule.

DISCUSSION

The isolation of water soluble biologically active compounds from Cannabis sativa is not novel (5,6), but the finding of water soluble material, which can be easily prepared from the raw material (16) and which has activity in reducing IOP is a new observation. The data appear different from the observations of West and Lockhart (7) in several aspects, primary of which is the solvent; West and Lockhart did not specify their solvent thus direct comparisons are difficult. Unlike their preparation which was tested in dogs and man, however, neither the crude material extract nor the more purified material we prepared had any activity when applied topically to the rabbit or primate cornea (8,16). The material prepared by Soxhlet extraction also produced a greater fall in IOP (Table 1) than that seen by West and Lockhart (7) although differences in the route of administration exist.

As our studies are progressing newer methods of preparation and isolation of biologically active compounds are being identified, including the use of dialysis of the crude water-extract which generates a material having similar biological activity as described in the present paper (8,16). The MDM used in the present study is not a single chemical species but a mixture, since it yields further subfractions following chromatography, which are active in reducing IOP (Deutsch and Zalkow, unpublished data). The MDM used was of high molecular weight (>12000 daltons) and contains a considerable amount of protein and carbohydrate (16).

The present studies have shown that a dose-related fall in IOP occurs when the MDM is given intravenously (Table 1), with concentrations as low as 0.005 mg/animal inducing effects which last as long as 15 hours. At high concentrations a general relaxation and antidiuresis is seen in the rabbit. The dose-response curve is the same in albino as in pigmented rabbits, indicating that there appears to be no discernible effect of melanin on the pharmacokinetics of MDM. The MDM is also quite stable at room temperature, since a solution, tested initially, showed a similar IOP activity after 3 weeks of storage.

High concentrations also elicit a two phase change in IOP, an initial hypertensive phase preceding a profound hypotensive phase, whereas lower doses only elicit the hypotensive phase. Tachyphylaxis was found to repeated systemic administration on a daily basis, even when the in-

itial dose was 0.01 mg/animal and successive doses of 0.1 mg/animal were given. Whether this is caused by the protein moeity of the MDM remains to be determined. The proteinaceous nature of the MDM is also seen following intravitreal injection of 1 mg, where an inflammatory response is noted after 36 hours. Lower concentrations also caused a proteinaceous aqueous humor to be found but the overall response was much less violent.

The initial hypertensive IOP change resembles that seen after prostaglandin (PG) release in, or topical application to, the eye (17-19). While PG's also cause a hypotensive IOP effect, the reduction is not as dramatic as found with the present MDM since the maximal fall in IOP after PG's was about 7 mmHg (to about 12 or 13 mmHg); in the present studies the maximal IOP fall was about 13 or 15 mmHg (to about 6 or 7 mmHg) or to values approximating episcleral venous pressure (20).

The antagonists alone had little effect on IOP. Phenoxybenzamine alone at 5 mg/kg caused a 25% fall in IOP between 4 and 7 hours but had no effect on the hypotensive effect of MDM (Table 2). The variability of the initial hypertensive phase provided some initial variability in the responses seen at the 1st, 2nd or 3rd hour but overall it appears that the adrenergic antagonists decrease the initial hypertensive response when used at 5 mg/kg (Table 2, compare low and high antagonist concentrations). This is adjudged from a consideration of the time-response profile of each individual animal and may be related to the IOP-reducing effects of the antagonists following intravenous injection (21). More certain is the lack of effect of these agents on the hypotensive effects of MDM. Topically applied timolol (0.5%) also had no effect on the IOP reduction induced by MDM, even when applied 1 hour before MDM and every hour thereafter up to 8 hours. The hourly regimen was chosen since it has been shown, in the rabbit, that timolol inhibition of isoproterenol-stimulated cAMP production by the iris-ciliary body was at a maximum at 30 min and decreased rapidly (22). Hexamethonium chloride, a ganglionic blocking agent, given at 6 hours after MDM was also without effect, and the response in the unilaterally ganglionectomized animals was identical in both the denervated and the contralateral normal eye. The data indicate that the ocular response to MDM does not occur through the adrenergic nervous system (Tables 2 and 3).

Indomethacin clearly inhibited the initial hypertensive response suggesting that a prostaglandin or related compound (23) participates in at least this phase of the response (Table 3). The hypotensive IOP effect and its time-course, is however, unaffected by indomethacin. That histamine is not involved was shown by the use of tripelennamine, which was without effect on either phase of the IOP change.

Topical administration of MDM to the normal eye revealed no effect on IOP due to the combination of the large molecular size, its water solubility and the permeability of, and lipid nature of, the epithelium. Even in eyes in which the epithelium had been removed no effect was seen. Neither intravenous administration to the monkey of as much as 20 mg/3 kg animal nor topical administration in the deepithelialized eye induced any IOP effect. The latter may reflect poor transcorneal penetration of this large molecule. The blood-aqueous barrier of the monkey eye is far less permeable to large molecular weight species than the rabbit eye, as would be anticipated from measurements of osmotic water permeability (24,25) and may explain the lack of effect in the monkey compared to the rabbit after intravenous administration. The intraocular penetration of MDM into the rabbit eye from circulation compared to that of the monkey may also be related to the involvement of prostaglandins, which break down the blood aqueous barrier in rabbit but have a much lesser role in the primate. The lack of response when MDM was given orally or by suppository must also be related to its high molecular weight and failure to cross the intestinal permeability barrier. Intraventricular administration was also without effect on IOP or body temperature.

The absence of blood pressure effects argues

against the IOP response being mediated by a systemic change. Since one of the side effects of high concentrations of MDM was an antidiuresis, antiogensin II was tested for its effects on IOP. Angiotensin has been shown in reduce IOP in the cat (26), but no effect was found in the rabbit at either 0.0025 or 0.005 mg/animal.

That the reduction in IOP is caused by a marked fall in aqueous humor flow rate, as determined by fluorophotometry is evident from Fig. 1. The reduction in aqueous humor flow rate correlates well with the fall in IOP with minima in both at about 9 hours after intravenous injection. Measurements by the inulin-dilution technique showed a 40% reduction in aqueous humor formation at 6 hours after intravenous injection of MDM which correlated with the fluorescein data. The underlying mechanism of the reduction in aqueous humor production is under investigation. The low rate of aqueous production, leading to the low IOP, may be partly responsible for the increase in aqueous protein content, although an increase is found during the initial hypertensive phase which may be PG-mediated.

Studies are being undertaken to determine which components of MDM are essential for its IOP-reducing effects. This is particularly pertinent in view of its profound effect in reducing IOP, since its large molecular weight predisposes against topical application. If only a small portion of the molecule is needed to elicit the response in IOP, then the material, or compounds derived from it, may be of potential clinical use in reducing IOP. The latter is only possible if the problem of tachyphylaxis is overcome, which could occur if the molecule can be made considably smaller and the protein moeity altered. A smaller molecule would also have greater potential for transepithelial and transcorneal penetration. As a research tool the water soluble Cannabis material is of interest due to its effect on aqueous humor inflow pathways and the possible identification of underlying mechanisms.

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Water soluble marihuana-derived material: pharmacological actions in rabbit and primate

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ABSTRACT

Further studies have been made with water soluble marihuana-derived material (MDM). Neither adrenergic, cholinergic, aldosterone, dopamine or serotonin antagonism affected the fall in intraocular pressure induced by MDM. Partial blockade was obtained with galactose, glucose, or mannose, but not arabinose, when the latter were given at intravenous concentrations of 1 gm/animal and MDM was given at 25 µg animal, suggesting that these sugars may be involved at the active site of the MDM glycoproteins. Dexamethasone was without effect on either intravenous or intravitreal MDM indicating that the MDM effect is not a non-specific response to a protein. A similar plant glycoprotein, larch arabinogalactan, at 200 µg/animal was without effect on intraocular pressure. Aqueous humor flow rate was increased 3 hours after MDM administration, a period corresponding to the intraocular pressure increase caused by MDM, and fell to 20% of control values when the fall in intraocular pressure occurred. Blood flow through the iris was increased at both one and six hours after intravenous MDM injection indicating a vasodilation which could contribute to the initial increase in intraocular pressure. Intravitreal injection of MDM in rabbit and rhesus monkey caused a fall in intraocular pressure only after a 24 hour delay: the unilateral response indicated that systemic metabolism was not required for activity and the delay was likely caused by the diffusion time to the ciliary processes from the mid-vitreal injection site. The changes in beta-receptors, adenylate cyclase and carbonic anhydrase in the ciliary processes are minimal indicating a possible vascular mechanism of action of MDM.

INTRODUCTION

We have previously described the isolation of water soluble compounds from marihuana which are active in reducing rabbit intraocular pressure (1). Extensive testing of this compound against several antagonists indicated that α- and β-adrenergic, prostaglandin, and histamine inhibitors were ineffective in altering the marked fall in intraocular pressure (IOP) found with marihuana-derived material (MDM) when the latter was given intravenously to rabbits (2). MDM was also unaffected by superior cervical ganglionectomy and was ineffective when given intravenously to rhesus monkeys. It remained to be determined, therefore, whether intrinsic pharmacological activity existed in the primate and whether the IOP response could be blocked in the rabbit in order to determine the mechanism of action of MDM.

Although these initial studies (1) provided some indication of the effects of MDM both in the eye and systemically, many questions remained concerning the mechanism of action. Since MDM has been identified as a glycoprotein (1), an approach which has proven effective in identifying the mechanism of action of other plant glycoproteins (3-5) was used in an attempt to make the same characterization of the present compounds. Additional studies have been made to establish the time course of the change in aqueous humor inflow.

MATERIALS AND METHODS

Drug preparation

Water soluble marihuana-derived material (MDM) was prepared as described previously (1,2) and made up fresh for each experiment in 0.9% saline. For intravenous injection 1 ml was used and for intravitreal injections the volume was 10 µl, the latter administered via a Gilman microliter syringe.

Animal testing

Rabbits. a) IOP measurements. Albino rabbits (2-3 kg) were restrained in canvas bags tied at the neck and the IOP measured with a calibrated Alcon Pneumatonograph. IOP was determined at -40 min, -30 min, 0 min, and at each succesive hour up to 7 hours on each test day. The -40 min reading

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was discarded and the -30 and 0 min values averaged to provide a time-zero value for each rabbit.

b) Antagonists. Cholinergic antagonists were atropine and scopolamine, with each administered as 1 drop of a 1% solution 1 hour before MDM administration. Other antagonists used were given intravenously: haloperidol lactate (dopaminergic antagonist) at a concentration of 2 mg/kg (6); methysergide (serotonin antagonist) at 5 mg/kg (7); chlorpromazine (adrenergic, cholinergic, serotoninergic and dopaminergic antagonist) at 1 and 10 mg/kg (8), spironolactone (aldosterone antagonist) at a concentration of 10 mg/kg (9), and yohimbine (α_2-antagonist) at 1 mg/kg (10).

c) Sugars. The sugars, either arabinose, galactose, glucose, or mannose, were injected intravenously at a concentration of 1 gram/animal 1 hour before injection of MDM (3-5). Intraocular pressure was measured hourly following the protocol outlined above. Control animals treated with sugar alone were always used in parallel with the experimental animals.

d) Steroid. A 1% solution of dexamethasone was applied topically to rabbit eyes at 2½ or 3 hour intervals for 1 or 2 days prior to intravenous or intravitreal injection of MDM.

e) Aqueous flow rate. Aqueous humor flow rate was determined using fluorophotometry (11), using an apparatus similar to that described by Waltman and Kaufman (12) attached to a Haag-Streit 360 slit lamp. Fluorescein (Fluress) was administered topically to the cornea every 2 min for 30 min, 3 hours before fluorescein concentrations were measured and 200 µg/ MDM was administered intravenously 4½ hours after the last fluorescein drop was given, at a time when both corneal and aqueous humor fluorescein concentrations were decreasing linearly. Control series were made to establish the most efficacious regimen of fluorescein application. The following solutions were used; 1 drop of Fluress (0.25% sodium fluorescein, 0.4% sodium benoxinate) every min for 30 min; 1 drop of Fluress every 2 min for 30 min; 1 or 5% fluorescein (in the presence or absence of 0.02% benzalkonium chloride) at either 1 drop every 2 or 5 min for 15 or 30 min and 10% fluorescein at 1 drop every 5 min for either 15 or 30 min. Aqueous humor flow rate was calculated using the approach of Yablonski et al (13), with a corneal volume of 80 µl and an aqueous volume of 180 µl. All animals were of approximately the same size (2 kg), thus this approximation allowed estimation of aqueous flow rate.

f) Intravitreal injections. Since intravenous injection of MDM was ineffective in rhesus monkeys (2), determination of any intrinsic pharmacological activity in this species required intravitreal injection. Studies were made, therefore, in the rabbit to delineate the quantity of MDM needed to elicit a response. These studies also allowed determination of whether systemic metabolism of MDM was needed to provide activity. Intravitreal injections were made unilaterally in the proptosed, locally anesthetized (tetracaine), rabbit eye following establishment of base line IOP. MDM was injected in various concentrations with vehicle injected controls run in parallel. Initially, IOP was measured hourly during the first 8 hours as well as between 22 and 30 hours but as the time course became evident readings were only taken at 2 hour intervals on day 1 after injection.

g) Blood flow determination. Regional ocular blood flow was measured using radiolabeled (^{85}Sr) 15 µ diameter microspheres using standard techniques (14-16). MDM was injected either 1 or 6 hours prior to blood flow determinations.

h) Blood pressure. Systemic blood pressure was measured by inserting a 22 gauge needle, connected to a Hewlett-Packard transducer and recorder, into the median ear artery under Cetacaine anesthesia (Cetylite Industries, Pennsauken, N.J.). Measurements were made at 0, 3 and 6 hours with either MDM or saline alone injected immediately after the "0" hour reading.

i) Plant glycoprotein. MDM has been characterized as a glycoprotein containing neutral sugars, including large amounts of arabinose, and protein (1); thus a similar plant glycoprotein, larch arabinogalactan (3), was also given

intravenously to rabbits at 200 μg/animal. IOP was determined as above for MDM alone.

Rhesus Monkeys. Rhesus monkeys (3 kg) were sedated with ketamine (30 mg) and received 1 drop of topical tetracaine prior to intravitreal injections of MDM using a 30 gauge needle under sterile conditions. The animals were then placed into restraining chairs (2, 17), which allowed suitable restraint yet the capability of measuring both IOP and total outflow facility following recovery from the 30-minute sedation period. A calibrated Alcon Pneumatonograph was used for both IOP and outflow facility determinations; a 10 gram weight was used for tonography and the latter was measured immediately after IOP measurements.

RESULTS

Rabbits.

a) IOP determinations. A dose response curve was generated as previously (2) and confirmed using a full spectrum of concentrations. Purer compounds have been identified subsequent to MDM isolation, some of which are active at concentrations as low as 1 μg/animal and which induce maximal (60%) falls in IOP at concentrations of 10 μg/animal. These purer compounds exert the same effects as MDM but are more active on a weight for weight basis. The data obtained with MDM, therefore, reflects that for purer compounds in terms of pharmacological activity. Isolation and characterization of a pure active compound has not yet been made.

b) Antagonists. None of the antagonists used affected the fall in IOP induced by MDM. None of the antagonists alone affected IOP, except the highest dose (10 mg/kg) chlorpromazine which significantly reduced IOP (8). This high dose of chlorpromazine lowered IOP to a point where further reduction was impossible. The maximal IOP fall and the time at which this occurred are shown in Table 1, and no change was seen compared to MDM alone even with 1 mg/kg chlorpromazine.

c) Sugars. The data obtained following sugar and MDM injection is shown in Table 2. Arabinose was ineffective in affecting the fall in IOP irrespective of the MDM concentration (25, 50 or 200 μg/animal). Neither glucose, galactose or mannose had any effect on the MDM-induced fall in

Table I: Antagonists which failed to affect MDM in rabbit.

				Antagonist															
MDM Concn (μg)	Time zero IOP	None		Time zero IOP	A 1%	Time zero IOP	Sc 1%	Time zero IOP	M 5	Time zero IOP	H 2	Time zero IOP	C 1	Time zero IOP	C 10	Time zero IOP	Sp 10	Time zero IOP	Y 1
0	19.7 ±0.4	31.2 ±5.3	C	20.7 ±0.6*	19.2 ±7.3*	20.3 ±0.3*	11.1 ±2.4*	-	-	21.1 ±1.8+	4.3 ±7.6+	26.0 ±0.3+	15.7 ±2.5+	24.2 ±0.4+	40.0 ±6.4+	20.9 ±1.6*	19.1 ±7.4*	23.4 ±0.8	+11.4 ±4.0
			E	21.3 ±0.6*	15.4 ±8.2*	19.5 ±0.4*	13.7 ±3.6*	-	-	-	-	-	-	-	-	-	-	-	-
50	19.8 ±0.7	28.4 ±9.3	C	20.9 ±0.8	28.4 ±9.3	20.4 ±1.8	36.3 ±10.2	-	-	-	-	-	-	-	-	-	-	-	-
			E	21.3 ±0.6	32.6 ±2.7	20.7 ±1.6	43.1 ±6.9	-	-	-	-	-	-	-	-	-	-	-	-
100	20.1 ±0.6	47.4 ±7.4		-	-	-	-	20.6 ±0.6	43.9 ±3.7	19.3 ±1.1	43.9 ±3.7	23.9 ±0.3+	34.6 ±3.6+	19.8 ±0.9	50.9 ±5.8	-	-	-	-
200	20.2 ±0.5	47.0 ±6.2	C	19.9 ±0.7	45.7 ±2.0	19.8 ±0.6	57.0 ±2.0	-	-	-	-	-	-	-	-	22.5 ±0.5	46.6 ±4.0	26.2 ±0.2	45.3 ±2.0
			E	20.9 ±0.7	49.3 ±6.6	19.8 ±0.8	56.7 ±4.6	-	-	-	-	-	-	-	-	-	-	-	-

The data represent percentage fall in IOP from time zero value (given in mmHg) at 6 hours after MDM administration (or antagonist administration at an MDM concentration of 0) was given intravenously. A, atropine; S; scopolamine; M, methysergide, 5mg/kg; H, haloperidol,2mg/kg; C, chlorpromazine, 1 or 10mg/kg; Sp, spironolactone, 10mg/kg; Y, yohimbine, 1mg/kg. Atropine and scopolamine were given topically; methysergide, haloperidol and chlorpromazine were given intravenously. For topically administered drugs the values are given for both treated eye (E, treated with topical antagonist) and control eye (C, non-antagonist treated). All values are the mean ± SEM of 6 animals except where indicated, *, n=3, +, n=4. No fall in IOP after antagonist plus MDM was significantly different from that with MDM alone.

Table 2: Effects of intravenous sugars on IOP after MDM in rabbit.

MDM concentration (μg)	Time-zero IOP	None	Time-zero IOP	Arabinose	Time-zero IOP	Galactose	Time-zero IOP	Glucose	Time-zero IOP	Mannose
0	-	-	27.2 ±0.9(4)	8.7 ±3.3(4)	28.1 ±0.3(4)	7.5 ±3.5(4)	28.9 ±0.8(4)	4.3 ±1.2(4)	26.9 ±0.8(4)	+1.5 ±5.1(4)
25	19.7 ±0.4(6)	31.2 ±5.3(6)	18.9 ±0.7(6)	33.9 ±3.1(6)	20.3 ±0.9(6)	22.6 ±3.6(6)*	21.2 ±0.6(6)	21.2 ±3.0(6)*	20.4 ±0.4(6)	18.2 ±3.0(6)*
50	19.8 ±0.7(6)	28.4 ±9.3(6)	17.2 ±0.5(6)	43.5 ±4.2(6)	18.2 ±0.4(4)	34.1 ±3.3(4)	21.0 ±0.2(4)	30.2 ±5.1(4)	16.7 ±0.8(4)	33.1 ±5.0(4)
100	20.1 ±0.6(6)	47.4 ±7.4(6)	-	-	21.7 ±0.8(4)	40.0 ±6.2(4)	-	-	-	-
200	20.2 ±0.5(6)	47.0 ±6.2(6)	24.1 ±0.3(6)	50.9 ±2.5(6)	24.4 ±0.5(6)	46.2 ±3.4(6)	24.6 ±0.4(6)	49.9 ±3.4(6)	22.2 ±1.9(6)	52.4 ±2.7(6)

The numbers represent the mean ± SEM of the percentage fall in IOP 6 hours after the time zero value (given in mmHg). The numbers in parenthesis are the numbers of animals. *, $P < 0.05$ compared to "no sugar" data.

Table 3: Effect of dexamethasone on IOP after MDM in rabbit.

MDM concentration (μg)	n	Route of administration	Steroid	Time-zero IOP (mmHg)	Maximal % fall in IOP	Time of maximal fall in IOP (hrs)
100	6	intravenous	T	20.1 ± 0.5	33.9 ± 2.2	5
			N	20.1 ± 0.9	34.0 ± 4.1	6
150	6	intravenous	T	21.8 ± 1.3	32.3 ± 4.1	6
			N	21.8 ± 1.1	34.2 ± 4.4	7
200	6	intravenous	T	19.8 ± 0.4	35.4 ± 3.2	6
			N	20.0 ± 0.8	35.2 ± 4.2	6
0	10	intravitreal	E	21.9 ± 0.1	16.6 ± 4.0	29
			C	19.1 ± 1.1	17.0 ±10.8	29
0.1	6	intravitreal	E	21.6 ± 0.7	41.6 ± 6.8	28
			C	20.3 ± 1.1	15.5 ± 8.5	28

Paired eyes were either T, treated with steroid; or N, not treated with steroid. Fall in IOP is the maximal percentage fall from the time zero value (given in mmHg) at the time shown. MDM concentration of 0 was saline control. All values are the mean ± SEM of the percentage fall in IOP. For intravitreal injections, both eyes were treated with steroid and only one (E) received MDM while the other (C) was the control eye. n, number of rabbits. * indicates significantly different from paired eye, $p < .01$.

IOP at MDM concentrations at or above 50 μg/animal. At 25 μg MDM/animal, however, galactose, glucose and mannose induced partial blockade at the MDM-induced fall in IOP.

d) Steroid. Dexamethasone pre-treatment alone caused only a slight (16%) fall in IOP. When MDM was given intravenously (Table 3) the fall in IOP was similar in both non-treated and steroid-pretreated eyes, and the fall in IOP in both eyes was similar to that seen in the absence of steroid. Intravitreal injection of MDM was also unaffected by steroid pretreatment (compare line 5 of Table 3 with Table 4).

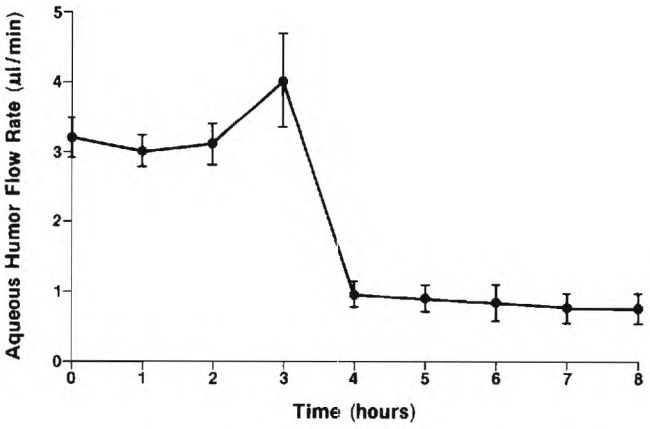
e) Aqueous humor flow rate. The rate of aqueous humor flow was calculated after various regimens of fluorescein application. The most effective regimen was 1 drop of Fluress applied every 2 min for 30 min; other regimens and solutions did not provide as high a corneal or aqueous fluorescein concentration.

The aqueous flow rate is shown in Fig. 1., indicating that the flow rate remains virtually unchanged over the first 2 hours after intravenous injection of 100 μg MDM/animal before increasing by about 30% at 3 hours. This increase was not statistically significant. The aqueous flow rate

Table 4: Effect of intravitreal MDM on rabbit IOP.

MDM concentration (µg)	n	Time-zero IOP		Time (hours) 2	4	6	24	26	28	30
0	6	22.3 ± 0.7	C	7.1 ± 8.3	0.0 ± 3.5	0.0 ± 3.5	4.7 ± 1.2	13.9 ± 3.3	16.2 ± 8.0	8.1 ± 4.6
	6	22.8 ± 0.8	E	+1.7 ± 0.5	8.4 ± 7.2	1.7 ± 0.5	9.6 ± 0.7	17.5 ± 2.6	20.7 ± 0.6	6.4 ± 10.8
0.01	6	20.9 ± 2.3	C	8.9 ± 2.9	2.6 ± 5.6	4.6 ± 4.1	+1.5 ± 22.0	0.2 ± 15.7	1.2 ± 16.3	8.8 ± 21.2
	6	23.7 ± 1.8	E	6.3 ± 8.7	4.8 ± 10.7	15.0 ± 8.4	27.9 ± 8.6*	38.1 ± 8.4*	36.7 ± 8.0*	38.6 ± 13.1*
0.1	6	19.9 ± 0.8	C	11.5 ± 7.8	16.5 ± 6.0	18.4 ± 8.7	15.3 ± 5.9	8.2 ± 8.1	12.6 ± 1.8	13.9 ± 3.9
	6	19.3 ± 1.1	E	6.2 ± 3.6	21.3 ± 7.2	17.0 ± 5.6	51.6 ± 6.1*	47.8 ± 5.3*	50.2 ± 4.9*	52.8 ± 3.5*
1	6	19.4 ± 0.7	C	7.9 ± 7.5	1.1 ± 6.0	4.6 ± 6.2	6.7 ± 5.3	1.0 ± 6.4	10.0 ± 7.8	6.3 ± 5.0
	6	20.1 ± 0.8	E	14.3 ± 2.7	9.4 ± 2.4	12.8 ± 3.2	43.7 ± 4.3*	52.5 ± 1.3*	50.7 ± 1.5*	43.8 ± 2.8*

MDM concentration (µg/eye) injected intravitreally in 10 µl. Values given are mean ± SEM of percentage fall in IOP from the time zero value (except where a + precedes the value, when this indicates an increase in IOP). Time-zero IOP value in mmHg. Time is hours after injection. C, non-injected eye; E, injected eye. Vehicle alone was used when MDM was 0. * indicates significantly different from paired eye, $p < .02$.



Aqueous humor flow rate as a function of time after intravenous MDM administration.

Values are the mean ± SEM of 6 animals (12 eyes) at each time shown.

then decreased markedly to a statistically significant maximal fall of 80% between 5 and 7 hours. This fall in flow rate correlated with the IOP at this time (14.2 ± 0.8 mmHg at 5 hrs after MDM, $p < .01$ compared to time zero value). The increased flow rate at 3 hours appears to be correlated with the already documented increase in IOP seen at a similar time after the administration of at least 100 µg MDM (2).

f) Intravitreal injections. The data obtained after intravitreal injection of MDM is shown in Table 4. Little change in IOP was found during the first 22-24 hours, but thereafter the IOP fell rapidly to low levels equivalent to those found after intravenous administration except that the fall in IOP was unilateral. The magnitude and duration of the fall in IOP was dependent upon the administered dose. In one group of 6 animals with 100 µg/eye the IOP was followed for up to 144 hours and the percentage fall in IOP of the injected eye from base line was 33.5 ± 1.2% and in the contralateral paired control eye was 4.3 ± 3.6% at 144 hours, indicating the longevity and unilateral nature of the response.

g) Blood flow determinations. The data indicate that iris blood flow is increased at both 1 and 6 hours after intravenous MDM, but the flow is unchanged in other ocular tissues (Table 5) compared to controls. The iris vessels were substantially dilated and with blood flow being the same in other tissues a greater blood flow occurred through the whole eye.

h) Blood pressure. Systemic blood pressure was found to decrease during the day in control animals by about 14 mmHg (Table 6) and a lesser change occurred in MDM-treated rabbits. No change was observed in these conscious animals which could contribute to the massive fall in IOP after MDM.

i) Plant glycoprotein. Intravenous larch arabinogalactan had no effect on rabbit IOP at a concentration of 200 µg/animal. Thus it is

Table 5: Regional ocular blood flow in rabbit after intravenous MDM.

Tissue	Blood flow (mg blood/mg tissue/min)		
	Control	1 hour	6 hours
Iris	1.29 ± 0.39	3.84 ± 0.84*	3.32 ± 0.49*
Scraped ciliary processes	2.37 ± 0.47	3.67 ± 0.69	3.15 ± 0.28
Retina	0.39 ± 0.08	0.22 ± 0.05	0.07 ± 0.02*
Choroid	19.83 ± 2.60	35.86 ± 7.87	22.57 ± 2.79

Values are mean ± SEM for 6 animals in each group. *, $P < 0.02$.

Table 6: Rabbit blood pressure after MDM

	Mean Blood pressure (mmHg)	
Time (hr)	Control	Experimental
0	73.8 ± 5.8 (6)	63.4 ± 1.9 (15)
3	62.5 ± 3.5 (6)	53.9 ± 2.9 (15)
6	59.8 ± 3.0 (6)	57.4 ± 2.0 (13)

Experimental animals received 100 µg MDM after the "0" hour reading and control animals received 1 ml saline. Values are the mean ± SEM; numbers of animals in parenthesis.

unlikely that a gross similarity of chemical structure or simply an osmotic effect of a large molecule in the circulation plays a role in reducing IOP. MDM, therefore, appears unique in its ability to reduce IOP.

Rhesus monkeys. Intravitreal injection of 0.01 µg MDM/eye resulted in a rise in IOP for the first 24 hours followed by a pronounced fall in IOP which lasted for approximately 3 days. Injections of 0.001 µg MDM/eye caused only a fall in IOP which was 50% at its maximum at 36 hours, and lasted for up to 54 hours. No change in total outflow facility occurred during either the rise or fall in IOP (Table 7). At neither concentration was the total outflow facility (mean value 0.58 µl/min/mmHg) markedly changed.

DISCUSSION The progressive sub-fractionation of MDM has identified a series of compounds which appear to be closely related, thus MDM is a mixture rather than a single chemical. As purification has proceeded, the activity of the materials, in terms of IOP-reducing capability per unit mass, has increased such that some compounds cause a fall in IOP up to 60% with a concentration of 1 µg/animal (unpublished data). The pharmacological profile is unchanged, however, since those more purified compounds thus far tested show the same action as MDM. Pharmacological data obtained with MDM, therefore, is relevant to subsequently purified compound(s), although purification of a single chemical structure has not been achieved to date.

The experiments reported here concern MDM and its collective pharmacological activity. The studies with atropine and scopolamine indicate that blockade of the cholinergic (muscarinic) receptors in the eye has no influence on the time course or magnitude of the pressure fall induced by MDM. Neither dopaminergic (haloperidol and chlorpromazine), adrenergic (yohimbine), adrenergic and cholinergic (chlorpromazine) nor serotoninergic (methysergide and chlorpromazine) antagonism affected the MDM-induced fall in IOP.

Spironolactone, an aldosterone antagonist, was also without effect, indicating that the mineralocorticoid did not interact with MDM. Neither haloperidol nor methysergide alone induced a change in IOP, but a high concentration of chlorpromazine alone caused a fall in IOP of up to 30% which lasted for about 6 hours which correlates with previous observations (8).

The studies with preloading of sugars, which has proven successful in other studies in identifying the mechanism of action of active glycoproteins (3-5), indicated that mannose, glucose and galactose were effective in causing partial blockade of the MDM effect of IOP, at least at low concentrations of MDM. Arabinose had no effect even at low MDM concentrations. Because MDM is a mixture it is difficult to assign a particular blocking effect to one sugar, and it appears that the active components of MDM have either mannose, glucose or galactose at a site which contributes to the pharmacological activity of MDM. Specific glycoproteins of MDM may contain only one of the sugar groups but these and other common features remain to be identified.

To determine whether or not the ocular hypotensive response was a non-specific inflammatory reaction, dexamethasone, a steroid known to enter the rabbit aqueous humor with ease (18), was administered for 2 days prior to MDM injection by either intravenous or intravitreal routes. No blockade was found to intravenous or intravitreal MDM administration. This confirms our previous observation (2) that boiled MDM failed to induce an effect on IOP. The effect on IOP appears to be a specific response and not a non-specific response to a foreign protein. Confirmation of this effect is seen with the absence of an inflammatory reaction with low concentrations of intravitreal MDM. The specificity of the fall in IOP to this particular arabinogalactan (MDM) is indicated by the failure of the IOP to respond to larch arabinogalactan.

Aqueous humor flow rate has been determined at hourly intervals following the intravenous, but not intravitreal, administration of MDM. Previous studies (2) began measurements at 5 hours and correlation was found between the decrease in aqueous humor flow rate and IOP, but the IOP is known to increase at early times (1-3 hours) after injection and it was important to determine whether or not the aqueous flow rate followed the same time course as IOP. The data obtained (Fig. 1) indicate that the ocular flow rate is virtually constant for 2 hrs after MDM administration before rapidly increasing by about one third and then declining rapidly to about 20% of control values at 4 hours through 8 hours. The IOP was recorded only at 5 hours in the present experiments and was significantly decreased (14.2 ± 0.8 mmHg), but the usual pattern of IOP (2) would have been expected at the dose of MDM used in these experiments. The increase in IOP at 1-3 hours after intravenous MDM may be associated with the increase in aqueous humor formation, as well as a change in ocular blood flow (Table 5). A change in ocular blood volume can cause a marked change in IOP (19).

Dilation of the renal vascular bed has been noted in the dog after MDM administration (G.C. Bond, personal communication), and it is possible that a similar change occurs in the rabbit. The blood flow measurements with radiolabeled microspheres indicate an initial iris vessel dilation at both 1 and 6 hours with other tissues showing no change compared to controls. The marked suppression of aqueous humor inflow at later times could mask any vascular effect on IOP.

Intravitreal injections of MDM produced a markedly different response from that seen after intravenous injection. With the latter, the fall in IOP occurred within 3 - 4 hours after administration but after intravitreal administration the fall in IOP was delayed up to a maximum of 24 hours. The unilateral response observed indicates that the effect was not due to systemic metabolism but rather a local response within the eye. This was reconfirmed by the data obtained after unilateral intravitreal injection in the rhesus monkey (Table 7). The cause of the delay in both species, however, remains undetermined at this time. It is surprising that the intravenous

Table 7: Effects of intravitreal MDM on rhesus monkey IOP.

Concentration		n	Time (hours)									
			0	6	24	26	28	30	48	50	52	54
0.01 µg	C	3	31.7 ±1.6	28.7 ±1.3	31.3 ±3.8	29.7 ±2.9	25.7 ±0.7	27.0 ±3.0	30.3 ±2.9	27.7 ±1.4	25.0 ±0.6	24.0 ±4.0
	E	3	36.3 ±1.9	30.3 ±4.7	47.0 ±3.0	34.2 ±10.4	20.2 ±4.9	17.7* ±1.7	15.0* ±5.0	13.3* ±4.4	11.7* ±2.8	12.0* ±2.5
0.001 µg	C	3	27.7 ±0.3	28.7 ±0.3	27.7 ±0.9	25.0 ±0.6	26.3 ±1.4	29.5 ±1.2	27.3 ±1.8	28.7 ±1.4	26.5 ±1.6	27.0 ±1.0
	E	3	32.2 ±2.0	32.7 ±1.2	16.7 ±6.6	16.3 ±6.7	15.3* ±6.7	16.0* ±4.9	17.3* ±7.3	16.7* ±7.3	15.3* ±6.1	16.7* ±5.3

C, control, paired eye; E, experimental, MDM-injected eye; n, number of eyes. Values are the mean ± SEM of the absolute IOP in mm Hg. * indicates significantly lower than paired eye at any time, $p < .05$.

administration of as little as 25 µg MDM/animal, or 0.17 µg/ml plasma, (with the plasma volume of a 3 kg rabbit being 150 ml) results in a fall in IOP with a short delay while the direct intraocular injection of MDM has a delay period with an average of about 22 hours. Whether this is related to the distribution of MDM in the eye and the time needed to reach a sufficiently high MDM concentration at the ciliary processes remains to be determined. It is known, however, that a small molecule such as sucrose, when injected intravitreally takes about 4 to 20 hours ($t_{1/2}$=15 hr) before high levels are observed in the aqueous humor (20). Thus, anteriorly directed diffusion of MDM, a much larger molecule (20,000 - 40,000 daltons) than sucrose, would be slower and the delay in response compared to intravenous MDM could be due to the fact that intravascular material could reach the ciliary processes much earlier than intravitreal MDM. A dose-related response occurs to intravitreal MDM, and the longevity of the fall in IOP appears related to the amount injected. IOP falls as long as 4 days were encountered with as little as 1 µg/eye with the fall in IOP still being about 48%, and at 3 days after 0.1 µg/eye an IOP fall of 42% was found.

The rhesus monkey data indicate that MDM has pharmacological activity in the primate when given intravitreally, and it is most likely that intraocular penetration, as discussed previously (2) is the reason for the difference between this data and intravenous administration. The lack of effect on total outflow facility indicates that the mechanism of action in the primate resembles that in the rabbit and that some component of aqueous inflow is severly compromised by MDM action.

Preliminary studies indicate that the MDM effect is not related to carbonic anhydrase since large amounts of MDM (up to 10 mg) had a minimal effect on the enzyme (T.H. Maren, personal communication), and no change in adenylate cyclase (by direct assay with ^{32}P-ATP on membrane fragments), or increase in responsiveness to β-agonists and guanyl nucleotides was found in rabbit iris-ciliary body (T.W. Mittag, personal communication). Furthermore, neither cerebrospinal fluid (CSF) production, nor CSF pressure nor arterial pressure in either cats and rabbits are affected by MDM given by intraventricular (25 µg/ml), intraperitoneal (250 µg/kg) or intravenous (1 mg/kg) routes (B.P. Vogh, personal communication). All the above experiments used MDM concentrations which were well in the range of inducing a substantial fall in IOP when given intravenously.

Despite the array of antagonists which have been employed (2, and Tables 1, 2 and 3) against MDM, the mechanism of action remains uncertain.

MDM reduces intraocular pressure by reducing aqueous humor formation and whatever component of aqueous is affected it is markedly impaired. The present data differ from that seen with cholera toxin (21) where intravitreal (0.26 μg) and intra-arterial (2.1 μg) injections cause a decrease in net aqueous flow by 50% in 8 hours and doubling of blood flow to the anterior uvea at 8 to 13 hours. Cholera toxin stimulates adenylate cyclase by 2.2 -fold and cyclic AMP production by ciliary processes by 7.4 times (21). Outflow facility has also been found to increase after intravitreal cholera toxin (22). MDM does not increase outflow facility, does not cause an increase in adenylate cyclase, appears to act exclusively on aqueous formation, but does decrease IOP by large amounts although the time course is quite different from that of cholera toxin.

The mechanism of action may reveal alternative pathways by which aqueous humor entry into the eye can be modified, and the effect in the primate offers the possibility that some component of the large molecule may be identified which could be capable of inducing a fall in IOP. The absence of a change in adenylate cyclase, -receptor response or other biochemical indices with MDM, such as seen after catecholamine or cholera toxin and which reflects an epithelial cellular response to these drugs, suggests that some vascular component of aqueous humor inflow is being affected. Such a change occurs despite the lack of a large effect on measured blood flow, since perhaps some previously unidentified regulatory component is affected by MDM. Furthermore, the failure of known receptor antagonists to affect the response induced by MDM suggests that MDM may not be acting at a specific recognition site but at another locus of action such as a cell membrane or by induction of a secondary response.

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Marihuana-derived material: distribution and effects after systemic administration

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ABSTRACT

Studies have been made in an attempt to elucidate the mode of action of water-soluble marihuana-derived material (MDM). MDM lowers intraocular pressure (IOP) at systemic dose levels greater than 5 µg/rabbit by reducing aqueous humor inflow. Blood pressure, body temperature, and PO_2 remain constant despite the wide variation in IOP caused by high dose levels of MDM, viz. an initial hypertensive phase followed by a hypotensive phase. Blood PCO_2 and pH, however, both decrease with 1 mg MDM/rabbit indicating an acidosis which may partially explain some of the fall in IOP caused by MDM at this high dose level. Low doses of MDM (50 µg/animal), however, induce no such changes in systemic chemistry, illustrating the absence of an MDM effect which can explain the greater than 50% fall in IOP. Repeated injections of MDM on a weekly basis indicate a sequentially reduced effect on IOP. MDM, when incubated in vitro for 6 hours with saline, aqueous or vitreous, always induced a fall in IOP; incubation in these media for 24 hours, however, reduced the capacity to induce an IOP decrease. When aqueous or vitreous was removed from animals which had received intravitreal injections of MDM 24 hours previously (thus, at a time when the IOP in these animals was low) and was reinjected intravitreally into fresh recipient rabbits, the IOP fell in the recipients with aqueous, but not vitreous. Only when high doses of MDM (≥ 2 mg) were given systemically to a donor rabbit was any evidence obtained of a fall in IOP in recipient rabbits at short times after the donor injection (< 10 min); at greater times after the donor injections whole blood or serum from donor rabbits failed to elicit a fall in IOP in recipient animals. These data indicate that, in vivo, MDM is bound or metabolized rapidly in rabbits when MDM is given systemically.

INTRODUCTION

It is now well established that either intravenously or intravitreally administered water-soluble marihuana-derived material (MDM) causes a marked reduction in intraocular pressure (IOP) in the rabbit (1,2). In rhesus monkeys, effects are only seen after intravitreal injections (2). Typical dose levels required to induce a fall in IOP of about 50% are: systemically, 1 to 5 µg/animal (1,3), and intravitreally, 0.001 to 0.01 µg/eye (2). This material, which is a high molecular weight glycoprotein (ca.5 x 10^5 daltons) (3,4), is, therefore, extremely potent, cannot be blocked by conventional antagonists (2,3), and its mode of action remains an enigma. Despite several attempts to discern the mechanism by which such a large reduction in IOP is induced, it has proven impossible to identify either systemic or ocular changes which are responsible.

In a further attempt to identify the processes which may impinge on the effects of MDM and to further discern its mode of action, a series of experiments were performed. These were directed at attempts to recover MDM at various times after systemic or ocular administration in order to determine whether MDM was binding to blood or serum constituents and to examine blood gas, as well as blood pressure, responses to various doses of MDM.

MATERIALS AND METHODS

Several types of experiments were performed using adult albino rabbits weighing between 2 and 3 kg. MDM was obtained from Mexican marihuana, supplied by the University of Mississippi Research Institute for Pharmaceutical Sciences under the auspices of the National Institute on Drug Abuse. Portions of leaves and small stems were macerated in a Waring blender with warm water (55-60°), the resultant liquid filtered, and the residue reextracted in water. Following centrifugation, dialysis and lyophilization, the samples were used (3,4).

Group I. Blood Pressure

Conscious rabbits were placed in canvas bags which were tied at the neck and blood pressure (BP) measured, via a heparinized 22 gauge needle and PE50 cannula inserted into the median ear artery, using a calibrated Hewlett-Packard transducer and recorder. Cetacaine spray was used as a topical anesthetic for needle insertion. The cannula remained inserted throughout the experiment and was flushed every 30 min with 1 ml of a 50% heparin-50% saline mixture. IOP and mean arterial BP were measured hourly over the course of 6 hours following initial pre-treatment measurements. IOP was measured using an Alcon Pneumatonograph. Values of IOP and BP for both experimental (received MDM in saline) and control (received equivalent volume of saline) groups were compared for each time interval within groups.

Group II. Blood Gases

Arterial blood samples were taken from a cannula in the median ear artery. Frequently in this series of experiments, BP was also measured hourly as described above. Blood (500-600 µl) was collected in pre-heparin rinsed 1 ml tuberculin syringes which were capped and stored in an ice bath until duplicate analyses were made using a Radiometer acid-base analyzer and a blood micro system. Analyses were usually made within 10 minutes of sampling. A control sample was taken before any treatment, either MDM in saline or saline alone, was administered. A small volume of blood was expelled from the syringe before placement of the sample in the blood-gas analyzer. Values of pH, PCO_2, and PO_2 were determined for each sample. The pH was calibrated against precision buffer solutions and PCO_2 calibrated using humidified gas mixtures with accurately known CO_2 concentrations. Measurements were made at 37°C. In several experiments, body temperature was also measured using a calibrated YSI tele-thermometer and a rectal probe. A small amount of 5% lidocaine ointment was administered prior to the probe insertion. The probe remained in place for 30 sec before the temperature was measured. Either a 1 mg or a 50 µg dose of MDM was administered.

Group III. Repetitive Injection Experiments

A group of rabbits was injected intravenously on day 1 with MDM in saline and the IOP recorded hourly for 6 hours after establishment of a time-zero baseline using -30 and 0 min readings which were averaged. The same rabbits received identical injections and determinations of IOP at weekly intervals over the next 5 weeks.

Group IV. Incubation Experiments

Experiments were performed in order to determine whether MDM activity was retained after incubation with saline, bovine serum albumin solutions, whole blood, serum, aqueous humor or vitreous humor. The MDM concentration was adjusted to provide a dose of 50 µg/rabbit irrespective of the solution. In some cases, incubation of MDM with the solution was performed at various times and temperatures.

Group V. Reinjection Experiments

This series was performed in two parts: a) systemic reinjection of blood, or serum and, b) intravitreal reinjection of aqueous or vitreous.

a) Rabbits were given MDM systemically and, at various times, whole blood was removed via a cannulated carotid artery in an animal anesthetized with xylazine and ketamine. Blood for whole blood reinjection was collected in a heparinized container, and blood for serum reinjection was collected in non-heparinized tubes, allowed to clot and subsequently centrifuged. Either blood or serum from the MDM donors was then reinjected into other groups of rabbits and their IOP monitored over several hours. Because of the dilution of MDM in the donor rabbits, high doses were administered which, after appropriate dilution in the body water, would provide concentrations in the reinjected whole blood or serum of approximately 50 µg/animal. The latter concentration has been repeatedly shown to induce a large fall in IOP upon initial intravenous injection (1-3).

b) In other studies, rabbits were injected

Table 1.
Effects of 1 mg MDM fractions on rabbit blood pressure and intraocular pressure

Times (Hr)		MDM # 1 BP	MDM # 1 % Change in IOP	MDM #2 BP	MDM #2 % Change in IOP
0	C	75.0 ± 2.1			
	E	77.7 ± 2.6		86.8 ± 6.1	
1	C	68.3 ± 3.2	-1.0 ± 4.8		
	E	64.7 ± 4.7	+23.6 ± 8.8*	73.1 ± 8.1	+24.1 ± 5.4*
2	C	68.0 ± 2.1	-8.4 ± 3.8		
	E	59.0 ± 6.8	-10.3 ± 6.0	55.1 ± 9.7	-0.3 ± 6.5
3	C	70.2 ± 0.8	-6.9 ± 4.0		
	E	56.2 ± 5.0*	-22.3 ± 10.2*	52.9 ± 9.2 (11)	-37.3 ± 7.1*
4	C	69.3 ± 2.3	-2.0 ± 5.2		
	E	59.6 ± 4.3 (5)	-39.4 ± 9.8*	64.7 ± 5.3 (11)	-48.8 ± 3.0*
5	C	71.5 ± 2.7	-1.0 ± 4.2		
	E	62.2 ± 4.2 (5)	-59.2 ± 8.9*	66.4 ± 6.3 (10)	-53.3 ± 4.2*
6	C	68.9 ± 2.7	-0.2 ± 5.7		
	E	58.5 ± 4.6 (4)	-70.3 ± 4.8*	66.1 ± 8.2 (7)	-55.5 ± 5.9*

C = control animals; E = experimental animals. MDM given intravenously at 1 mg per animal in 1 ml heparinized saline; controls received 1 ml heparinized saline. BP in mmHg is the average arterial pressure. Percent change in IOP calculated from the time-zero value (average of -30 min and 0 min readings). Values are the mean ± SEM; * $P < 0.05$ compared to the paired animal for MDM #1 and to pre-treatment values for MDM # 2. Number of animals = 6 for MDM #1, and 12 for MDM #2, unless indicated otherwise in parenthesis.

intravitreally (2) with high concentrations (1 µg) of MDM in 10 µl saline before subsequent removal of aqueous or vitreous and its reinjection intravitreally into other animals. The IOP was measured in the initial MDM donors in order to determine when the fall in IOP occurred for aqueous and vitreous retrieval purposes and, in the reinjected animals, to determine whether or not the MDM, or a metabolite, remained active. The placement of 1 µg into 2 ml of vitreous gives a final concentration of 0.5 µg/ml. Reinjection of 50 µl of this sample provides a dose of 25 ng which has repeatedly been shown to induce large unilateral IOP reductions in rabbit (2).

Other experiments were performed in which aqueous or vitreous was removed from fresh eyes and MDM was incubated with these fluids for either 6 or 24 hours at 37° C prior to injection unilaterally into rabbit eyes (the contralteral eyes received an equal volume of saline). The injected dose was 1 µg MDM in 10 µl of aqueous or vitreous.

RESULTS

Group I. Blood Pressure

The data are shown in Table 1 for two derivatives, where it can be seen that although the BP tends to fall in both control and experimental animals for MDM #1, there is no statistically significant difference between the saline- and MDM-treated groups. When the response of each animal is examined, there is no correlation between those animals with large falls in BP and those animals with the greatest falls in IOP. This maintenance of blood pressure occurs de-

Table 2.
Effects of 1 mg MDM fraction on rabbit blood gases, pH and body temperature

Time (Hr)		PCO_2	PO_2	pH	T(°C)
0	C	32.5 ± 2.0	77.3 ± 2.5	7.47 ± 0.03	39.3 ± 0.2
	E	35.5 ± 2.2	73.3 ± 5.0	7.46 ± 0.02	38.9 ± 0.5
1	C	31.0 ± 1.5	77.0 ± 2.5	7.46 ± 0.02	39.7 ± 0.2
	E	25.6 ± 1.8*	68.1 ± 12.8	7.45 ± 0.01	40.8 ± 0.4
2	C	29.9 ± 1.0	79.9 ± 2.5	7.44 ± 0.02	39.5 ± 0.3
	E	19.3 ± 1.6*	79.6 ± 7.1	7.40 ± 0.06	39.6 ± 0.5
3	C	27.0 ± 1.3	81.4 ± 3.7	7.45 ± 0.02	39.5 ± 0.3
	E	16.7 ± 1.5*	90.5 ± 5.4	7.37 ± 0.02*	39.6 ± 0.6
4	C	27.9 ± 1.0	80.0 ± 1.5	7.45 ± 0.02	39.8 ± 0.3
	E	17.5 ± 2.1*(5)	84.9 ± 1.7 (5)	7.40 ± 0.01 (5)	40.3 ± 0.4
5	C	26.6 ± 0.9	81.1 ± 1.5	7.44 ± 0.02	40.1 ± 0.4
	E	17.9 ± 1.8*(4)	88.5 ± 1.9*(4)	7.40 ± 0.03 (4)	40.5 ± 0.4 (5)
6	C	26.0 ± 0.8	80.6 ± 1.2	7.45 ± 0.01	40.2 ± 0.3
	E	19.3 ± 2.7*(4)	87.8 ± 0.8*(4)	7.37 ± 0.03*(4)	40.1 ± 0.6 (4)

C = control, received 1 ml heparinized saline; E = experimental, received 1 mg MDM in 1 ml heparinized saline. PCO_2 and PO_2 given in mmHg. Values are the mean ± SEM of 6 animals, unless indicated otherwise in parenthesis. * $P < 0.05$ comparing control and experimental rabbits.

spite wide variations in IOP caused by 1 mg of MDM #1, from an increase of 24% at 1 hour, to a statistically significant fall of 60% or 70% at 5 to 6 hours. With 1 mg of MDM #2, no parallel control series were performed; but, nevertheless, there is no statistically significant change in BP compared to pre-drug values despite a statistically significant rise in IOP at 1 hour and a fall after 3 to 6 hours.

Group II. Blood Gases

With an MDM dose of 1 mg/rabbit, body temperature and PO_2 remained constant or increased (PO_2) during the course of the 6 hour experiment. PCO_2, on the other hand, although decreasing even in control animals relative to pre-test values, decreased to values between 75% and 80% of the controls in 1 mg MDM-treated animals (Table 2). Concurrent with the fall in PCO_2, there is a statistically significant fall in pH of MDM-treated animals at 3 and 6 hours. The IOP (see Table 1), however, showed an increase at 1 hour which was followed by a prolonged and marked fall from 3 to 6 hours.

Systemic injection of 50 µg, which is a dose level known to cause a substantial fall in IOP of over 40% (1,2, also Table 3), caused no changes in BP or blood pH, PO_2 or PCO_2. Thus, MDM was without effect on parameters which could explain, at least in part, the fall in IOP at this dose level.

Group III. Repeated Injections

Injection of MDM in successive weeks, at a dose level sufficient to induce a marked fall in IOP on day 1 of week 1, caused a lessened fall of IOP on successive weeks (Table 4). By weeks 4 and 5, the response to MDM is almost eliminated, and the results resemble those obtained from a saline injection only (compare Table 1).

Another series of 8 animals received 25 µg of MDM intravenously on day 1 and showed a maximum fall in IOP of 33.2 ± 6.0% at 6 hours. Injection the following day of 10 µg of MDM resulted

Table 3.
Effects of 50 µg MDM fraction on rabbit blood gases, pH, BP and IOP

Time (Hr)		PCO_2	PO_2	pH	IOP	BP
0	C	33.4 ± 1.2	70.2 ± 4.5	7.44 ± 0.01	28.0 ± 1.1	83.2 ± 1.6
	E	30.6 ± 1.7	75.9 ± 4.2	7.46 ± 0.02	29.7 ± 1.1	81.8 ± 2.5
1	C	28.0 ± 1.1	74.6 ± 3.6	7.44 ± 0.01	-2.0 ± 5.1	76.0 ± 2.3
	E	26.5 ± 1.3	75.9 ± 2.1	7.44 ± 0.01	7.2 ± 4.8	72.5 ± 1.9
2	C	29.2 ± 0.9	70.5 ± 2.6	7.43 ± 0.01	-2.8 ± 5.6	75.7 ± 1.8
	E	22.5 ± 2.1*	79.0 ± 3.1	7.43 ± 0.01	2.8 ± 10.4	76.8 ± 3.8
3	C	25.1 ± 0.9	73.8 ± 2.8	7.44 ± 0.01	-1.4 ± 4.3	78.8 ± 1.4
	E	23.7 ± 1.8	74.4 ± 3.4	7.43 ± 0.01	-13.8 ± 10.5	77.7 ± 3.7
4	C	25.7 ± 0.8	72.4 ± 1.0	7.44 ± 0.01	2.1 ± 5.2	78.8 ± 1.4
	E	23.9 ± 1.1	79.1 ± 3.1	7.43 ± 0.01	-36.7 ± 6.9*	76.5 ± 2.5
5	C	25.4 ± 0.9	76.0 ± 3.0	7.45 ± 0.01	-5.8 ± 5.4	76.7 ± 3.3
	E	25.1 ± 0.8	77.7 ± 3.5	7.43 ± 0.01	-44.9 ± 5.4*	72.5 ± 1.6
6	C	25.1 ± 1.0 (5)	77.1 ± 3.2	7.44 ± 0.01	-2.0 ± 3.5	78.8 ± 2.2
	E	25.4 ± 1.0 (5)	76.5 ± 2.4 (5)	7.42 ± 0.01 (5)	-48.6 ± 3.9*(5)	67.0 ± 3.7* (5)

C = Control, received 1 ml heparinized saline; E = experimental, received 50 µg MDM in 1 ml heparinized saline. PCO_2, PO_2 and BP given in mmHg. 0 hour IOP in mmHg; all succeeding values are % change. Values are the mean ± SEM of 6 animals, unless indicated otherwise in parentheses. * $P < 0.05$ comparing control and experimental rabbits.

in a maximum fall in IOP of 27.6 ± 4.7% at 5 hours. Thus, the time course and overall magnitude of the response was similar on both days. Whether this is a consequence of a lower dose administered on day 2 remains unknown. MDM, at 25 µg, in fresh rabbits caused a maximal IOP fall of 30.0 ± 3.8% at 4 hours. Thus, the induced fall in IOP is similar to that caused in the repetitive injection series after the first injection.

Group IV. Incubation Experiments

MDM incubated in saline for 1 hour at 37°C, when injected intravenously into rabbits (50 µg/animal), induced a fall in IOP of 23.0 ± 3.5% (n=8) at 5 hours (see also Table 1). MDM was then incubated with fresh whole blood or serum for 1 hour at 37°C or for 20 hours at room temperature before intravenous injection into rabbits. The MDM dose was 25 µg or 50 µg/animal but no IOP fall was noted from either whole blood or serum in the presence or absence of MDM. When incubated for 1 hour at 37°C, MDM in either 100 mg% or 1000 mg% bovine serum albumin (BSA) induced maximal falls in IOP of 49.4 ± 3.9% (n=8) and 39.6 ± 3.2% (n=8), respectively, when given at 50 µg MDM/animal in 1 ml of BSA solution. Intravenous injection of 1 ml of 1000 mg% BSA solution alone induced a maximal fall in IOP of 14.2 ± 3.7% (n=8). When MDM was incubated for 1 hour at 37°C with either fresh aqueous or vitreous, the IOP fell by 24.5 ± 5.5% and 35.7 ± 4.0%, respectively, upon intravenous injection of these solutions at 50 µg MDM/animal indicating that MDM was unaffected by exposure to aqueous or vitreous.

The data with incubation of MDM with whole serum (vide supra) raised the possibility that some fraction of the serum was responsible for

Table 4.
Effects of weekly injections of MDM in one group of rabbits

	Time (Hr)						
	1	2	3	4	5	6	7
Week 1							
% Change in IOP	+9.5 ± 4.1	+0.3 ± 4.8	-11.3 ± 5.0	-31.6 ± 3.8*	-32.8 ± 3.7*	-34.2 ± 3.9*	-35.8 ± 3.8*
Week 2							
% Change in IOP	-8.1 ± 2.7	-4.9 ± 4.0	-9.1 ± 3.0	-9.9 ± 1.7	-17.4 ± 1.9*	-20.8 ± 2.6*	-18.6 ± 2.5*
Week 3 (n = 7)							
% Change in IOP	-1.5 ± 1.5	-2.5 ± 4.9	-5.5 ± 4.0	-13.5 ± 3.3	-19.2 ± 4.0*	-17.4 ± 3.9*	-15.7 ± 4.7*
Week 4 (n = 5)							
% Change in IOP	-2.1 ± 4.6	-6.2 ± 3.8	-9.3 ± 6.2	-10.7 ± 3.0	-0.5 ± 6.0	-7.1 ± 4.2	-7.1 ± 3.3
Week 5 (n = 5)							
% Change in IOP	-5.8 ± 1.3	-15.1 ± 4.1	-7.7 ± 2.8	-6.1 ± 3.3	-8.9 ± 3.7	-6.2 ± 2.4	-9.1 ± 2.3

Repeated injection of 50 µg MDM were given weekly. Percent fall in IOP calculated from time zero value of IOP (average of -30 min and 0 min readings prior to injections). Values are the mean ± SEM. n = 8 unless otherwise indicated in parenthesis. * P < 0.05 compared to control, pre-treatment values.

MDM binding or chemical modification. To study this in greater detail, 10 ml of serum was dialyzed against 1000 ml distilled water at room temperature for 24 hours. Both the retentate and dialysate were freeze-dried, and reconstituted in 10 ml of heparinized saline containing 500 µg MDM and incubated at 37°C for 1 hour. Eight rabbits were injected with either reconstituted dialysate or retentate containing sufficient MDM to give 50 µg/animal. Both solutions caused a marked fall in IOP; serum retentate, 29.6 ± 3.4% at 6 hours and serum dialysate, 37.7 ± 2.3% at 6 hours.

Group V. Reinjection Experiments

a) Systemic Readministration. MDM was given to rabbits at a systemic dose of 2 mg. Given distribution in only the vascular system of the donor rabbits (due to the large molecular size and the short time after injection), this should result in 1 ml of blood containing about 10 µg, thus reinjection of 2.5 ml should result in approximately 20 to 25 µg of MDM being administered to the recipient animals. Experiments performed with whole blood and plasma retrieval at 7 minutes after the initial 2 mg MDM injection resulted in a fall in IOP in recipient rabbits which was a maximum of 36.8 ± 3.8% at 6 hours after reinjection. At 2 hours after 2 mg MDM, retrieval of whole blood or serum and subsequent reinjection into other animals failed to induce any change in IOP over 7 hours (2 series, maximum fall in IOP, 8.9 ± 3.1% at 1 hour [n=8] and 7.2 ± 3.3% [n=8] at 5 hours). Similar results (maximal fall in IOP, 9.4 ± 2.6% [n=8] at 3 hours) were obtained when blood was taken at 6 hours (the time of maximal IOP fall in the initially-injected donor rabbits). At 24 hours after the initial injection, reinjection of 2.5 ml of serum from donor animals into fresh rabbits caused a maximal fall in IOP of 24.1 ± 2.4% at 4 hours after injection. Recovery of whole blood and serum from rabbits at 8 minutes after receiving 10 mg MDM caused a maximal IOP fall of 54.8 ± 4.0% at 6 hours after reinjection into recipient rabbits.

b) Intraocular Readministration. Incubation of MDM for 6 hours with fresh aqueous or vitreous, when injected into vitreous of fresh rabbits at a dose of 1 µg MDM/10 µl aqueous or vitreous, initiated a large unilateral fall in IOP in the MDM-injected eye (Table 5). Incubation for 24 hours caused a much reduced response

Table 5.
Effects of 1 µg MDM when incubated with aqueous or vitreous

Eye	Injection	Time	% Fall in IOP
OS (n = 4)	Saline	24 hr.	5.5 ± 14.1
		42 hr.	13.9 ± 11.5
		95 hr.	15.6 ± 14.6
OD (n = 4)	MDM incubated in aqueous for 6 hr	24 hr.	22.6 ± 4.1*
		42 hr.	49.6 ± 3.3*
		95 hr.	44.8 ± 4.5*
OS (n = 4)	Saline	24 hr.	14.2 ± 9.8
		42 hr.	16.9 ± 6.1
		95 hr.	12.4 ± 2.7
OD (n = 4)	MDM incubated in vitreous for 6 hr	24 hr.	38.4 ± 3.6*
		42 hr.	39.6 ± 2.3*
		95 hr.	40.0 ± 4.3*

MDM was incubated with fresh aqueous or vitreous for 6 hours and injected intravitreally in 10 µl aqueous or vitreous into rabbits. Percent fall in IOP was calculated from time-zero values; values are the mean ± SEM. * $P < 0.05$ compared to paired, saline-treated eye.

comparing saline-injected and MDM-injected eyes (Table 6). The 6 hour incubation indicated that neither aqueous nor vitreous alone influenced the effect of intravitreal MDM. Intravitreal MDM at 1 µg/eye induced a large fall in IOP in the recipient eye, while the contralateral saline-treated eye showed only a minimal fall in IOP (2).

A further experiment was performed in which 1 µg of MDM in 10 µl saline or 10 µl saline alone was injected intravitreally into paired eyes of recipient rabbits. At 26 hours after these injections, when the donor rabbit IOP was at a minimum, both aqueous and vitreous were collected from these MDM-injected eyes and 50 µl of the collected aqueous or vitreous reinjected intravitreally and unilaterally into fresh eyes (saline was injected into the contralateral eyes). Given an ocular volume of 2 ml, the injection of 1 µg would give 0.5 µg/ml or 0.025 µg/50 µl; reinjection of 0.025 µg per eye should induce a marked unilateral fall in IOP (2). The data in Table 7 indicate that reinjection of aqueous and vitreous that had received saline alone caused a substantial fall in IOP which tended to recover to near normal values after 49 hours. With aqueous from eyes previously injected with MDM, however, the IOP stayed low for a considerably longer period of time. Little difference was seen between the vitreous reinjection using either saline or MDM-treated vitreous.

DISCUSSION

Several water-soluble fractions of material isolated from marihuana have proven effective in reducing IOP in rabbit and dog when given systemically (1-3) and in the primate and rabbit when given intravitreally (2). Recent studies on purer fractions have indicated that the protein content is relatively small and that the carbohydrate fraction is correspondingly large (3,4). Chemical analyses have delineated the composition of these fractions with considerable accuracy (3). Three active and potent fractions of high (5×10^5 daltons) molecular weight have

Table 6.
Effects of 1 μg MDM when incubated with aqueous or vitreous

Eye	Injection	Time	% Fall in IOP
OS (n = 4)	Saline	24 hr.	25.7 ± 7.5
		48 hr.	31.1 ± 5.8
		142 hr.	13.9 ± 5.5
OD (n = 4)	MDM incubated in aqueous for 24 hr	24 hr.	30.1 ± 4.9
		48 hr.	37.6 ± 1.8
		142 hr.	18.7 ± 5.8
OS (n = 4)	Saline	24 hr.	21.6 ± 7.2
		48 hr.	32.8 ± 6.7
		142 hr.	15.8 ± 8.8
OD (n = 4)	MDM incubated in vitreous for 24 hr	24 hr.	38.9 ± 6.0
		48 hr.	50.3 ± 2.7*
		142 hr.	21.1 ± 14.2

MDM was incubated with fresh aqueous or vitreous for 24 hours and injected intravitreally in 10μl aqueous or vitreous into rabbits. Percent fall in IOP calculated from time-zero values; values are the mean ± SEM. * $P < 0.05$ compared to paired, saline-treated eye.

Table 7.
Effect of injection of aqueous or vitreous from previously injected rabbits

Eye	Injection	Time	% Fall in IOP
OD (n = 4)	Aqueous from eye previously injected with sterile saline	26 hr	30.1 ± 8.0
		42 hr	23.6 ± 4.9
		49 hr	4.2 ± 7.6
OS (n = 4)	Aqueous from eye previously injected with 1 μg MDM	26 hr	7.7 ± 2.4
		42 hr	26.8 ± 11.4
		49 hr	26.7 ± 13.4*
OD (n = 4)	Vitreous from eye previously injected with sterile saline	26 hr	16.3 ± 2.8
		42 hr	18.2 ± 4.5
		49 hr	5.2 ± 5.6
OS (n = 4)	Vitreous from eye previously injected with 1 μg MDM	26 hr	10.0 ± 8.0
		42 hr	19.4 ± 3.8
		49 hr	0.9 ± 6.6

Percent fall in IOP calculated from time-zero values; values are the mean ± SEM. * $P < 0.05$ compared to paired eye.

been identified (4). MDM #1 and #2 are two of these fractions.

Earlier studies of MDM effects in rabbits indicated that BP was unaffected when compared to saline-treated animals, but measurements were made only intermittently, using low MDM doses

and with cannula reinsertion at appropriate times. Despite the use of a topical anesthetic, the manipulative procedures could evoke disturbance of the normal pattern of BP; thus, the present studies were designed to overcome these difficulties. A continually implanted cannula served as the means by which BP was monitored frequently. Despite the injection of a high concentration of MDM (1 mg compared to 50 µg; the latter is the dose which normally reduces IOP by at least 40%), there is no statistically significant effect on BP (Table 2). Such a high concentration of MDM was used in order to accentuate any systemic effects which might be induced. With a dose of 50 µg MDM, it was verified that no change in BP occurred (Table 3), despite an over 40% fall in IOP. The IOP during the first 6 hours after injection of a high concentration (1 mg) of MDM undergoes a marked rise by as much as 25% within 1 hour, followed quickly by a fall to about 60% of the baseline IOP within 3 to 5 hours (Table 1). No correlation exists, therefore, between BP and IOP during the course of the IOP change and the IOP fluctuations must be caused by effects other than BP. Dose levels of 1 mg and greater per animal have been previously shown to cause relaxation of conscious rabbits sufficient that muscle tone is lost and the animal becomes flaccid (1).

Body temperature and PO_2 remain constant during the first 6 hours after administration of 1 mg MDM. PCO_2 and pH, however, show a decrease, particularly of PCO_2. Although no changes in blood gases occurred which might correlate with the increase in IOP at 1 hour, there does appear to be some relationship between PCO_2 and the fall in IOP. Acidosis (5) and reduction in PCO_2 (6) have been shown to reduce IOP by effects on aqueous inflow (5). Previous studies have linked the fall in IOP with a change in either pH (5,6) or PCO_2 (7). Our studies indicate non-significant changes in blood pH (except at 3 and 6 hours) but significant falls in PCO_2 from the second hour after MDM-administration. The present data, therefore, indicate that PCO_2 changes alone can account for some of the the fall in IOP. Induced metabolic acidosis reduced the IOP for at least an hour (5) but no comment was made on the duration of the fall in IOP, although the maximal fall was reached at 30 min and rose gradually during the next 30 min but not regaining the original value. Aqueous inflow was decreased by over 50% (5) with large systemic changes in pH, which correlates with our findings of an 80% fall in aqueous inflow with MDM. It is possible, therefore, that at least part of the explanation for the effects of large doses of MDM lies in the induction of a loss of CO_2, and a degree of acidosis. Intravenous HCl is known to reduce the IOP by approximately 30% and pH by 0.16 units after 30 min, while the reduced aqueous inflow was measured at 1 hour (5). MDM is known to reduce IOP by at least 50 to 55% by 6 hours after administration at the dose levels (1 mg) employed here (Table 1), thus acidosis caused by the fall in PCO_2 could be responsible for approximately 25% of the total IOP fall caused by MDM at this dose level. This value is obtained from a consideration of the fall in IOP for a given change in PCO_2, based upon earlier studies (5,6). The maximum pH difference between control and MDM-treated animals was a non-significant 0.08 units, while the maximal PCO_2 difference was 10.4 mmHg. The rate of change of PCO_2 has been linked to the change in IOP, as well as the retention of low IOP in the face of normal values for PCO_2 and pH after a decrease in the latter parameters (7). The rate of change in the present experiments (Table 2) was relatively slow, although the amount of change (approximately 10 to 12 mmHg) in these other series (7,8) did cause a fall in IOP of about 35%.

A low dose level of MDM, 50 µg, which still induced a large (50%) fall in IOP (Table 3) caused no change in BP, pH or PCO_2. Thus, no relationship exists at lower MDM doses which still induce substantial changes in IOP, with

systemic blood gas chemistry, and other explanations must be sought to explain the fall in IOP.

Repeated injections of MDM into the same animals resulted in a decreasing effect with time, indicating a tachyphylaxis to MDM. The reasons for this effect remain undetermined, but the data confirm earlier findings after daily administration (1). It is surprising that even at weekly intervals that there is a tachyphylaxis since the IOP, after a single administration, returns to normal levels by between 28 and 36 hours at the dose levels employed in this series (50 ug/animal).

The incubation and reinjection experiments indicate several important facts concerning the behavior of MDM. First, the incubation at 37°C in saline in the presence or absence of BSA has no effect on its subsequent effect in the rabbit, although incubation with serum or whole blood inactivates the material. Secondly, incubation of MDM for 1 hour at 37°C with aqueous or vitreous with subsequent intravenous injection again has no effect on the MDM-induced response, indicating that neither aqueous or vitreous contain the mechanism for deactivation of MDM. It is unlikely that the lack of effect after incubation in whole blood or serum reflects binding since an unaltered response was elicited by MDM in a 1000 mg percent BSA solution. The absence of a response when MDM was incubated in whole blood or serum appears to reflect a change induced by some breakdown or modification of MDM. This must remain to be determined, however, since intravenous administration of MDM still causes alterations in IOP either in short-term (elevation at 1 hour) or long-term (decrease by 3-4 hours) *in vivo*. Thus, the *in vivo* data indicate that whatever effect blood or plasma has on MDM does not affect its pharmacological properties, whereas *in vitro* incubation with whole blood or plasma appear to render MDM pharmacologically inactive when injected into fresh rabbits. If metabolism occurs *in vivo*, an explanation must be sought for the inability of whole blood to perform this event *in vitro* with subsequent *in vivo* effects upon injection.

The results from reinjection of whole blood or serum from initial donor animals indicate that only at short time intervals after high doses of MDM injection are effects seen in recipient rabbits. High ($\geq$ 2 mg) dose levels presumably either have little opportunity to distribute in the available body water, are not bound or metabolized by whole blood or present such an excess of material to the donor system that binding/metabolizing loci cannot handle the load. Whatever the reason, there is also little question that at times when the donor rabbit IOP is either elevated (due to the high dose level) or depressed, there is no effect seen in rabbits which are recipients of donor whole blood or serum. There was a suggestion that recovery of blood at 24 hours did cause an effect when reinjected into fresh recipient rabbits.

Reinjection of aqueous from a previously MDM-injected eye into fresh recipient eyes did cause a reduction in IOP which was greater than that caused by aqueous from a contralateral saline-injected eye. There was no difference between vitreous recovered from either MDM or saline-injected eyes. It is possible that the MDM had either become bound to ocular tissues or passed from the vitreous into the aqueous since the latter pathway would be that which a material of this size would follow. The aqueous reinjection data indicate that MDM does indeed pass through the aqueous on its way out of the eye and that it remains active. Perhaps this is a consequence of the small amount of material necessary to induce a response when MDM is placed intravitreally. MDM which is neither metabolized, bound or consumed during passage from vitreous to aqueous is that which is retrieved and remains active upon reinjection. Perhaps the reason for the lack of vitreous effect is due to the loss of MDM in the time interval chosen for this experiment.

Overall, the present experiments show that MDM

is somehow removed from the vascular circulation within a very short time after intravenous injection because subsequent removal of whole blood or serum and reinjection into other animals does not reduce IOP in the recipients; that incubation in media other than whole blood or serum does not decrease activity, whereas incubation in whole blood or serum inactivates the material; and that, although high (1 mg) doses of MDM cause systemic acidosis, low dose levels (50 µg/rabbit) do not induce changes in blood gas chemistry indicating that this mechanism does not account for the fall in IOP.

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Polysaccharides from *Cannabis sativa* Active in Lowering Intraocular Pressure

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SUMMARY

Aqueous extraction of air dried Cannabis sativa *(marijuana) yields, after dialysis, a mixture of high molecular weight carbohydrate-containing components. This mixture has very potent intraocular pressure-lowering activity (antiglaucoma) when tested by intravenous injection into rabbits. Partial purification by DEAE-cellulose and gel filtration chromatography has yielded very active material (lowers intraocular pressure maximally at 1 μg/animal) with an estimated molecular weight of about 500 000. The active material contains mostly carbohydrate with a small amount of protein. Rhamnose, galactose and uronic acid are the major sugar constituents. The composition of components suggests that the active material is a pectic polysaccharide possibly derived from the cell wall.*

INTRODUCTION

We have discovered a novel activity for polysaccharides solubilized from dried leaves of *Cannabis sativa* by extraction with hot water. These

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carbohydrates, which have certain chemical and physical characteristics of pectic polysaccharides, lower intraocular pressure (IOP) when injected intravenously into rabbits. We have shown that these water-soluble materials lower IOP by a different mechanism (Green *et al.*, 1981, 1982) from certain cannabinoids (Green, 1982). Since the elevated IOP caused by glaucoma is a major cause of blindness, this polysaccharide material may have pharmaceutical applications. Further chemical investigations of these carbohydrates were therefore undertaken, and the results are reported in this paper.

EXPERIMENTAL

Extraction of *Cannabis sativa*

Samples of dried marijuana (primarily leaves and small stems of Czechoslovakian variety) were obtained from the University of Mississippi Research Institute for Pharmaceutical Sciences under the auspices of the National Institute of Drug Abuse. Portions were macerated in a Waring blender (3·8 litre capacity) with warm (55–60°C) water (eight times the weight of the sample). Optimum activity was obtained from samples blended at low speed (15 500 rpm) for 0·5–1·0 min. The macerated samples were filtered through cheesecloth under vacuum, and the residue was then re-extracted with warm water without further maceration. Extracts were centrifuged at 1340 *g* for 60 min; the supernatants were dialyzed against distilled water, lyophilized and tested for IOP-lowering activity.

Ion exchange chromatography

Crude, dialyzed extracts were lyophilized and then applied to a column (2·5 cm × 45 cm) of DEAE-cellulose equilibrated in 0·05 M Tris-HCl, 0·25 M NaCl, pH 6·7. The first 250 ml eluting under these conditions were pooled, dialyzed against water and lyophilized. Highly-coloured material was retained on the column.

Chemical analysis

The carbohydrate compositions of various samples were determined by gas chromatography of the alditol acetates using the method of Albersheim *et al.* (1967). Gas chromatography using flame ionization detection (FID) was performed with a 2 mm × 1·8 m column of SP 2340 and

a temperature programme beginning at 190°C for 5 min and then 5°C per min to 275°C. Helium was used as the carrier gas at a flow rate of 30 ml/min.

Total neutral sugar was determined by the phenol–sulphuric acid method of DuBois *et al.* (1956), using D-glucose as standard.

Partial amino acid analyses were performed on acid hydrolysates (6 M HCl, 18 h, 110°C *in vacuo*) by ion-exchange chromatography using post-column derivatization with ninhydrin.

Uronic acid content was determined by the method of Blumenkrantz & Asboe-Hansen (1973), using *o*-phenylphenol (Sigma) as reagent and galacturonic acid as the standard.

Protein content was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

Reduction of uronic acids

Uronic acids were reduced with $NaBH_4$ after activation of carboxyl groups with a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma), according to the method of Taylor *et al.* (1976), scaled to a 1 mg sample. The reduced polysaccharide, recovered by lyophilization instead of precipitation, was hydrolyzed, and the products were converted to alditol acetates for analysis as described above.

Gel filtration chromatography

A column (1·5 cm × 45 cm) of Fractogel TSK HW-65F (Merck) was equilibrated in 0·05 M $(NH_4)_2CO_3$, pH 7·8. Samples (100 mg) of DEAE-cellulose fractions were applied, and 1·4-ml fractions were collected. Aliquots of fractions were assayed for total carbohydrate, uronic acid, protein, neutral sugar, and IOP-lowering activity.

Chromatography on Fractogel TSK HW-65F was performed with three other solvent systems: 0·05 M sodium phosphate–0·2 M LiBr, pH 6·5; 4 M guanidine hydrochloride; and 0·5 M NaCl containing 0·005 M EDTA.

Affinity chromatography

A column containing Concanavalin A–agarose (Sigma) was equilibrated in solvent containing 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0·1 M NaCl. The pH

was adjusted to 5·3 with acetic acid. A sample of DEAE-cellulose fraction was applied in the same solvent, and the column was eluted with one column volume of equilibrating solvent. The column was then eluted successively with one column volume each of solvents containing 20 mM α-methyl-D-mannoside and 200 mM α-methyl-D-mannoside. The three fractions were dialyzed against distilled water, lyophilized, and analyzed for carbohydrate and IOP-lowering activity.

A column of *Ricinus communis* agarose (RCA-I, E-Y Laboratories) was equilibrated in 0·01 M Tris-HCl, 0·1 M NaCl, pH 7·65. A sample of active fraction 2 was applied in this buffer. The column was eluted with successive column volumes of starting buffer and buffers containing 20 mM and 200 mM β-methyl-D-galactoside. Each of the three fractions was dialyzed against distilled water, lyophilized, and analyzed for IOP-lowering activity.

Chromatofocusing

A column (1·0 cm × 30 cm) was packed with PBE 94 (Pharmacia) and equilibrated in 0·025 M imidazole-HCl buffer, pH 7·35. Polybuffer (Pharmacia) (5 ml), pH 4·0, was passed through the column, and then a sample of a DEAE-cellulose fraction in imidazole buffer was applied. The column was eluted with Polybuffer, pH 4·0, and the effluent was monitored at 280 nm. Fractions were collected, and aliquots were assayed for neutral sugar. The pH of the fractions was also monitored. Washing the column with 1 M NaCl removed material bound at pH 4·0.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to Gabriel (1971).

Pronase digestion

A 6·4 mg sample of a DEAE-cellulose fraction was dissolved in 1 ml of 0·15 M Tris acetate–0·0015 M calcium acetate, pH 8·0. Digestion was carried out at 37°C in the presence of toluene as an antibacterial agent for 72 h with five additions of pronase (Sigma Protease XIV). The total sample volume was then applied to the gel filtration column, and carbohydrate was monitored by the phenol–sulphuric acid assay.

Alkaline borohydride reaction

A 1·4 mg sample of an active fraction obtained from DEAE-cellulose chromatography was dissolved in 1·4 ml of 0·05 M NaOH containing 1 M $NaBH_4$ and heated at 50°C for 15 h. An aliquot was taken for IOP testing, and the remainder was applied to the gel filtration column. Fractions were monitored for carbohydrate by the phenol–sulphuric acid assay.

Intraocular-pressure screening

IOP measurements were made with an Alcon Pneumatonograph on both eyes of an adult rabbit. Baseline values were obtained on readings 30 min prior to and just before the time of intravenous injection of the sample to be tested. Seven hourly measurements were made after delivery of the sample. Measurements taken from four rabbits treated identically at the same time were averaged, and percentage changes in IOP are reported. Additional details of the assay have been described by Deutsch *et al.* (1981) and Green *et al.* (1981).

RESULTS AND DISCUSSION

All water extracts and subsequent fractions obtained from *Cannabis sativa* were tested for IOP-lowering activity by intravenous injection into rabbits (Green *et al.*, 1981; Deutsch *et al.*, 1981). In these assays, activity is expressed as the percentage fall in IOP after administering the sample compared with resting pressure. The maximum percentage fall is about 60%, at which point the IOP is equal to venous pressure.

Water extracts from *Cannabis sativa* were dialyzed and fractionated on DEAE-cellulose. Batchwise elution of DEAE-cellulose using 0·05 M Tris-HCl, pH 6·7, containing 0·25 M NaCl yielded an active material used for subsequent studies to characterize the active component. Attempted elution of extracts at lower ionic strength resulted in material becoming irreversibly bound to the resin.

The active fraction from DEAE-cellulose chromatography was analyzed for carbohydrate and protein content. Carbohydrate analysis of the active DEAE-cellulose fraction showed the presence of about 36% neutral sugar by weight (Table 1), and the relative proportions of

TABLE 1
Composition of Fractions (Weight Per Cent) Obtained from Water Extract of *Cannabis sativa*

Fraction	*Neutral sugar*[a]	*Uronic acid*[b]	*Protein*[c]
DEAE-cellulose	36	12	26
Fractogel			
1	19	3	38[d]
2	22	12	16[d]
3	22	10	23[d]

[a] Determined by phenol-sulphuric acid assay.
[b] Determined by *o*-phenylphenol assay.
[c] Determined by Lowry method.
[d] Determined by amino acid analyses.

TABLE 2
Neutral Sugar Composition[a] (Mol Per Cent of Total Neutral Sugar) of DEAE-Cellulose Fraction Before and After Carbodiimide-Activated Reduction of Uronic Acids

Sugar	*Without reduction of uronic acid*	*With reduction of uronic acid*
Rhamnose	14·2	13·1
Arabinose	31·1	22·5
Xylose	6·6	7·6
Mannose	3·8	5·1
Galactose	35·4	41·8
Glucose	11·0	9·9
Glucosamine	trace	trace

[a] Determined by g.c. analysis of alditol acetates.

neutral sugars were determined by gas chromatography of the alditol acetates (Table 2). The protein content of each fraction, as determined by the Lowry colorimetric test, and the uronic acid content, as determined by the *o*-phenylphenol colorimetric test, are also listed in Table 1.

The type of uronic acids present in the active material was ascertained by reduction of the acids to the corresponding neutral sugars by the method of Taylor *et al.* (1976). A sample of the DEAE-cellulose fraction was treated with a water-soluble carbodiimide and reduced with $NaBH_4$. Reduction of uronic acids was 98% complete, based on the colorimetric response of a control sample before and after reduction using the *o*-phenylphenol reagent. Recovery of material was ascertained by phenol–sulphuric acid assay before and after reduction; according to this method, virtually 100% of the material was recovered. The reduced sample was then hydrolyzed and subsequently converted to the alditol acetates in the normal fashion. The neutral sugar composition showed significant changes in the amounts of only two of the sugars, arabinose and galactose (Table 2). The arabinose content decreased by almost 9%. In our hands, the method used for the reduction of uronic acids has resulted in loss of arabinose in all samples tested, including standards such as gum arabic. The acidic conditions of the reduction may cause this loss of arabinose. The galactose content showed a 6% increase compared with the non-treated control. These results suggest that the predominant uronic acid present in the active sample is galacturonic acid. The degree of esterification of the uronic acids has not yet been determined.

An attempt was made to purify the DEAE-cellulose fraction further by means of the affinity of its carbohydrate moieties for Concanavalin A, a lectin which shows affinity for α-D-mannose and α-D-glucose residues. When a sample was applied to a column of Concanavalin A–agarose, however, the active component passed through the column unbound.

Chromatofocusing was used in an attempt to separate the components of the DEAE-cellulose fraction by their isoelectric points. The active material remained bound to the column and was only eluted with 1 M NaCl, suggesting that the material has an isoelectric point less than 4·0.

Separation of the DEAE-cellulose fraction on a column of Sepharose 6B showed the presence of very high molecular weight components which eluted at or near the void volume. Excluded material on this column would be expected to have a molecular weight near 1×10^6. A column of Fractogel TSL HW-65F, which fractionates polysaccharides in a molecular weight range of 1×10^4 to 1×10^6, was prepared in 0·05 M $(NH_4)_2CO_3$. A sample of the DEAE-cellulose fraction was

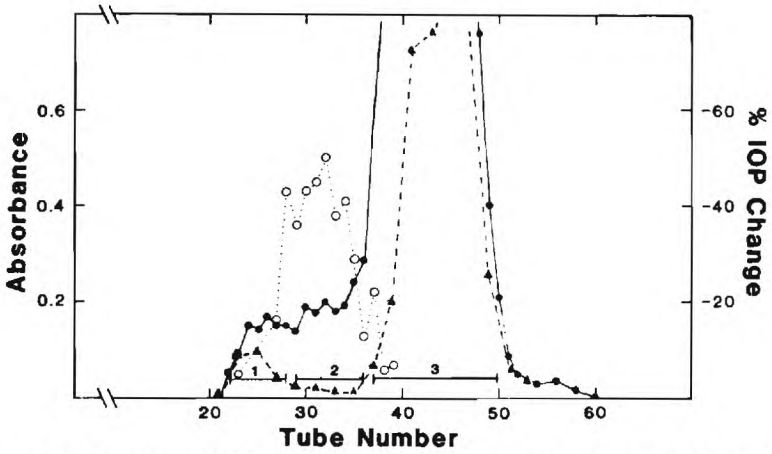

Fig. 1. Gel filtration of DEAE-cellulose fraction on Fractogel TSK HW-65F. A 1·5 cm x 45 cm column was equilibrated in 0·05 M $(NH_4)_2CO_3$, pH 7·7. Neutral sugar was monitored by phenol–sulphuric acid assay and absorbance at 490 nm was measured (●). Protein was assayed by the Lowry method, and absorbance at 660 nm was measured (▲). IOP was monitored as explained in the Experimental section (○).

separated on this column as seen in Fig. 1. The column effluent was monitored by the phenol–sulphuric acid colorimetric test for carbohydrates, and individual tubes were analyzed for neutral sugar and IOP-lowering activity.

Neutral sugar composition of the individual fractions of the Fractogel TSK HW-65F chromatography was determined by g.c. analysis of the alditol acetates. The results are plotted in Fig. 2. The most active fraction correlates with the neutral sugar composition which is highest in rhamnose and galactose as shown in Table 3 and indicated on Fig. 2.

The IOP-lowering activity for the individual fractions obtained after gel filtration chromatography as shown in Fig. 1 indicates that only those fractions in the intermediate area of the fractionation range exhibit activity. These tubes were pooled and designated fraction 2. Fractions 1 and 3 were also pooled as indicated in Fig. 1. The molecular weight range of the active fraction was estimated between 5×10^5 and 1×10^6 by calibrating the Fractogel column with a dextran sample of 5×10^5 average molecular weight. Attempts to assess the homogeneity and molecular weight of each fraction by conventional polyacrylamide

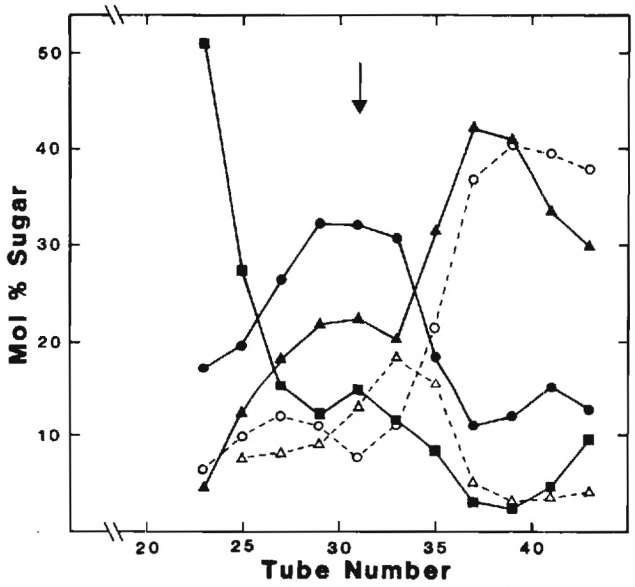

Fig. 2. Neutral sugar composition (mol% of total sugar) of individual fractions obtained from Fractogel TSK HW-65F chromatography of the DEAE-cellulose fraction. The sugars indicated are as follows: rhamnose (●); galactose (▲); glucose (■); arabinose (○); xylose (△).

TABLE 3
Neutral Sugar Composition[a] of Fractogel TSK HW-65F Most Active Fraction

Sugar	*Content (mol %)*
Rhamnose	32·2
Arabinose	7·6
Xylose	11·4
Mannose	5·0
Galactose	23·7
Glucose	15·6
Glucosamine	4·5

[a] Determined by g.c. analysis of alditol acetates.

TABLE 4
Neutral Sugar Composition[a] (Mol Per Cent of Total Neutral Sugar) of Fractogel TSK HW-65F Pooled Fractions

Sugar	*Fraction 1*	*Fraction 2*	*Fraction 3*
Rhamnose	19·8	27·9	12·7
Arabinose	9·8	9·9	38·1
Xylose	7·6	11·5	4·0
Mannose	10·6	6·1	4·5
Galactose	12·2	25·0	29·8
Glucose	27·2	14·1	9·5
Glucosamine	12·8	5·6	1·4

[a] Determined by g.c. analysis of alditol acetates.

TABLE 5
Partial Amino Acid Composition (Mol Per Cent) of Fractogel TSK HW-65F Pooled Fractions[a]

Amino acid	*Fraction 1*	*Fraction 2*	*Fraction 3*
Aspartic acid	12·0	11·6	13·7
Threonine	7·3	7·2	7·0
Serine	6·6	6·6	7·0
Glutamic acid	11·6	10·5	12·3
Glycine	10·6	9·4	9·9
Alanine	10·6	10·1	10·7
Valine	6·6	4·7	5·9
Methionine	1·2	1·1	1·2
Isoleucine	5·3	4·7	4·7
Leucine	12·7	17·9	8·4
Tyrosine	3·7	7·6	5·0
Phenylalanine	4·3	3·9	3·1
Histidine	1·7	0·3	7·9
Arginine	5·8	4·5	3·1

[a] Lysine was not resolved from ammonia. The amino acid analyzer was not equipped with a 440 nm channel for detecting proline and hydroxyproline.

gel electrophoresis (using 3·75% gel) were unsuccessful since the high molecular weight material would not enter the gel matrix. Summaries of the carbohydrate and partial amino acid analyses data for these fractions are given in Tables 4 and 5, respectively. A summary of the purification scheme routinely used and typical yields obtained for the isolation of active material is shown in Fig. 3.

The high content of galactose in the active fraction 2 suggested that further purification might be possible using the *Ricinus communis* lectin which specifically binds β-D-galactose. A sample of fraction 2 was applied to a *Ricin*–agarose affinity column which was successively eluted with the starting buffer, buffer containing 10 mM β-methyl-D-galactopyranoside and buffer containing 200 mM β-methyl-D-galactopyranoside. The sugar composition and IOP-lowering activity of the resulting three fractions are shown in Table 6. The bulk of the material and most of the activity eluted with the starting buffer. Again, the sugar composition of this active fraction shows a high percentage of rhamnose and galactose.

Since the high molecular weight of the active fraction poses problems in purification and characterization, several methods were

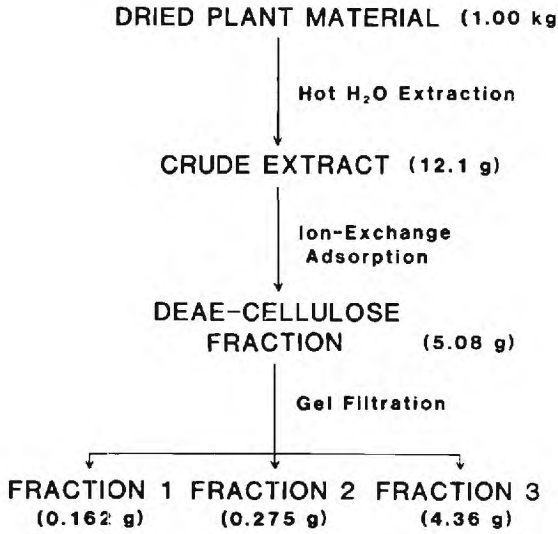

Fig. 3. Purification scheme for isolation of IOP-lowering active material from *Cannabis sativa*.

TABLE 6
Neutral Sugar Composition (Mol Per Cent of Total Neutral Sugar) Obtained from Ricin Affinity Chromatography of Fraction 2

Sugar	*Fraction*			
	Sample applied	*Unbound fraction*	*20 mM Methyl Gal*	*200 mM Methyl Gal*
Rhamnose	27·9	29·5	20·7	10·2
Arabinose	9·9	12·4	21·6	12·6
Xylose	11·5	7·8	3·5	9·4
Mannose	6·1	5·0	1·9	8·7
Galactose	25·0	24·0	43·8	31·5
Glucose	14·1	14·7	6·1	26·0
Glucosamine	5·6	6·7	2·3	1·6
IOP-lowering activity[a]	−47·6	−50·4	−12·8	−9·9

[a] 2 μg dose, 4–7 h average fall.

attempted to reduce the size of the material and yet retain activity. Two methods were tried, one which specifically cleaves amino acid linkages and one which cleaves *O*-glycosidic bonds.

A sample of the DEAE-cellulose fraction was digested extensively with pronase, a mixture of nonspecific proteases which digests peptides to free amino acids. The digested sample was applied to a gel filtration column. Compared with the original elution profile of the DEAE-cellulose fraction, the high molecular weight peaks shifted slightly to lower molecular weight. Also, the low molecular weight peak broadened. The activity profile, however, remained the same.

An active fraction obtained from gel filtration was treated with alkaline borohydride which hydrolyzes *O*-glycosidic bonds. This sample was polydisperse in molecular weight when reapplied to the Fractogel column and was not active.

Our work indicates that the water-soluble component from *Cannabis sativa* that is active in lowering IOP is of high molecular weight (between 5×10^5 and 1×10^6 daltons) and primarily a carbohydrate-containing material. From chromatofocusing studies, the isoelectric point of the active material appears to be less than 4·0. Rhamnose, galactose and

uronic acids are the major sugar constituents. The active material does not bind on columns of Concanavalin A or *Ricinus communis* lectin suggesting that α-D-glucose, α-D-mannose, or β-D-galactose are not present in bindable locations on the material. The composition of the components and its extraction from plant tissue by hot water suggest that the active (IOP-lowering) material is a pectic polysaccharide.

The high molecular weight of this material has posed problems both in separation procedures and in formulating a mode of action for the component. Chaotropic (0·2 M LiBr) and denaturing solvents (4 M guanidine-HCl; 0·5 M NaCl–0·005 M EDTA) have not affected the apparent molecular weight of the material as determined by gel filtration; the amino acid composition also does not show the presence of a large number of nonpolar residues that might enhance aggregation of several discrete components. Digestion with a nonspecific protease does not affect IOP-lowering activity, whereas extensive degradation of carbohydrate with alkaline borohydride destroys activity. These results in conjunction with the sugar composition data suggest that we might be dealing with a cell wall component (Aspinall, 1980; McNeil *et al.*, 1984).

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Marihuana-derived material: biochemical studies of the ocular responses

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ABSTRACT

Some biochemical factors of the iris-ciliary body of the rabbit have been examined for effects induced by water-soluble marihuana-derived material (MDM). Adenylate cyclase activity and sensitivity to β-adrenergic agonists were unchanged, as measured 4 hours after MDM administration in vivo. Magnesium-dependent and anion-sensitive, but not sodium-potassium, ATPase activities were inhibited 6 hours after MDM administration in vivo, although they were unaffected by in vitro incubation. Topical administration of a potent substance P antagonist had no effect on the time course or magnitude of intravenous MDM-induced ocular effects in rabbit. Intravenously administered sugars antagonized the effects of MDM on intraocular pressure. A variety of drugs which display a range of biochemical effects varying from beta-adrenergic receptor agonism, to alteration of glycoprotein residues were employed. None of the agents employed, ranging from cAMP modifiers to protein synthesis blockers, had any effect on the MDM-induced response. It is apparent that the mechanism underlying the ocular hypotensive effect of MDM does not reside in mediation through adenylate cyclase, ATPase or substance P, but rather through a mechanism mediated by terminal sugar moieties on the molecule. The data suggest that modification of the surface membrane glycoprotein residues on the ciliary epithelium can induce marked alterations in aqueous humor flow rate.

INTRODUCTION

Water-soluble materials derived from marihuana (MDM) have been shown to be highly effective in reducing intraocular pressure (IOP) in rabbits following intravenous injection (1,2). These materials have been chemically identified as a rhamnogalacturanon containing some protein (3,4), and detailed analyses of purified extracts have been made (4). Prior studies have indicated that many antagonists, including adrenergic agents, prostaglandin inhibitors, parasympathetic agents, and steroids (1,2), have failed to block the IOP-reducing effects of this material, and its mechanism of action has been elusive.

Biochemical studies of the iris/ciliary processes might provide information relevant to understanding of how MDM could exert such a profound effect on IOP. Studies were made, therefore, of the effects of MDM on the activities of adenylate cyclase and ATPases in the iris/ciliary body, and of the action of MDM in the presence of a substance P antagonist. Some of the biochemical techniques employed, although in vitro in nature, have been previously shown to reflect the effects of prior in vivo treatment regimens to various drugs (5-7). Further studies have also been made on the effect of amino sugars on the MDM-induced response, following the initial observation that sugars reduce the MDM effect in rabbits. A variety of other antagonists or modulators of protein and glycoprotein synthesis have been used in attempts to modify the response to MDM as it has become apparent that MDM appears to induce a fall in IOP by methods which have not previously been demonstrated in the eye (8). These modulators of biochemical events were chosen to alter the ocular status with regard to specific or generalized responses which could interact with MDM. Evidence is accumulating that MDM appears to act through effects on membranes within the eye, particularly those related to aqueous humor production (2,8).

MATERIALS AND METHODS

Adult albino rabbits, 2-3 kg, were used. MDM was used in either solid or liquid form (4). The solid form (MDM #1), although powerful in terms of induction of a fall in IOP was less potent than the more purified liquid form (MDM #2). The reason for the material being in liquid form was merely to enable very small μg quantities to be made accurately in small volumes (385 μg/1000 μl or 1.93 μg/5 μl), as volumetric measurements of

this size are more readily made than gravimetric measurements. MDM was always given intravenously. The experiments can be divided into five groups:

Group I - Adenylate Cyclase Activity

Experimental rabbits received 200 µg MDM #1 (in normal saline)/animal via an ear vein, and were killed 4 hours afterwards, when the IOP is minimal (1,2) (minimum IOP is reached at 4 hours but sustained for several more hours), while control rabbits received the saline vehicle and served as paired controls. Adenylate cyclase activities were determined on membrane fragments, using direct assay with ^{32}P-ATP, prepared from a treated and untreated pair. The methodology followed that of Mittag and Tormay (5) to determine the stimulation index, with control enzyme activities under each assay condition expressed as 100%. Tissues were collected from enucleated eyes and homogenized in Tris-buffer (5). Homogenates were centrifuged, the supernatant decanted, and the membranes resuspended in Tris-buffer. Drugs were added, as needed, after a 2 min incubation at 30°C, and preincubated for an additional 2 min before addition of substrate prior to a final 10 min incubation.

Group II - ATPase Activity

Treatment was made with MDM #1 either *in vivo* (200 to 400 µg/rabbit), or *in vitro* (5 to 12 µg per 20 mg protein). A parallel *in vivo* control series with saline injection was also employed. In all experiments, the iris-ciliary body was removed and shaken with 2 ml of Tris-buffer in a 5 ml glass bottle containing small glass beads (6). This suspension of epithelial cells was diluted to 10 ml, which gave approximately 200 µg protein/ml. With *in vivo* treatment, tissues were removed at 6 hours after injection; whereas, for *in vitro* studies, MDM was either added directly to the assay tubes or allowed to interact with the homogenate for 4 hours. The assay procedures for ATPase activity were those described previously (7) for the determination of Mg^{2+}, Na,K-stimulated, and anion-sensitive fractions, with incubation in a Tris-buffered solution at pH 7.95.

Group III - Substance P Antagonist

One of the responses to systemic MDM in rabbits is a slight rise in aqueous humor protein (1), and substance P may be a candidate for the mediator of some of the ocular responses to MDM. Substance P antagonist (D-Pro^2, D-Phe^7, D-Trp^9-SP) (9) was added topically to the right eye of conscious rabbits at either 0.1 or 10 µM in a 50 µl drop. MDM #1 was injected intravenously at 100 µg/animal 1 hour afterwards. IOP was measured hourly using an Alcon pneumatonograph, and the percentage change in IOP was calculated with reference to the mean of a 30 min and 0 min reading, prior to drug addition.

Group IV - Sugar Antagonism

Sugars were given intravenously in a volume of 2 or 3 ml at a dose of 1 gram/animal 1 hour prior to systemic injection of various doses of MDM #1 (5, 10, 25, or 50 µg/animal). A time zero IOP value was obtained and the IOP determined hourly for up to 8 hours. Frequently a parallel series of experiments was run with MDM alone for direct comparison with the sugar/MDM animals as the cost of the amino sugars was high, thereby precluding an independent series of amino sugar controls. Previous studies (2) have indicated that the intravenous injection of several sugars did not induce a fall in IOP.

Group V - Antagonists and Agonists

Several antagonists or agonists were used; their properties and doses are listed in Table 1. Theophylline was used both topically and intravitreally; isoproterenol was used in a 4% topical drop form (50 µl); guanosine 5'(-1,=-imido)triphosphate, sialic acid (N-acetylneuraminic acid), tunicamycin, and actinomycin D were all used intravitreally. All the preceding drugs were obtained from Sigma Chemical Company. SITS (4-acetamido-4'-isothiocyanato-stilbene-2-2'-disulfonic acid) (Gallard-Schlesinger Chemical Manufacturing Co.) and mannose-6-phosphate and yeast mannan (Sigma) were used both intravitreally and intravenously. Each drug was chosen for its ability to alter a specific primary biochemical event, although they may also induce secondary changes.

Table 1
Agonists and antagonists used against MDM #2 (5 µl [1.93 µg]/rabbit), their mode of action, dose level, and route of administration

Drug	Action	Dose	Route of Administration
Theophylline	Phosphodiesterase inhibitor (10)	10^{-2} M	Topical
		10^{-2} M	Intravitreal
Isoproterenol	Beta adrenergic agonist (11)	4%	Topical
Guanosine 5'-(β,α-imido)triphosphate	Cyclic AMP activator (12,13)	10^{-4} M	Intravitreal
Mannose-6-phosphate	Blocker of mannose-terminated glycoproteins (14,15)	10^{-3} M	Intravitreal
		10 mg	Intravenous
Sialic Acid	Antagonist of glycoprotein residues (14,16)	10^{-4} M	Intravitreal
Tunicamycin	Decreased glycosylation of proteins (17-19)	2 µg/ml	Intravitreal
Actinomycin D	Protein synthesis inhibitor (20)	10^{-5} M	Intravitreal
SITS	Anion transport inhibitor (21-23)	1.1 mg	Intravitreal
		83 mg	Intravenous
Mannan (Yeast)	Blockade of specific glycoprotein residues (15,16)	10 mg	Intravenous
		10 µg	Intravitreal

Numbers in parentheses are reference numbers to the effects of each compound.

The drug concentration chosen was based upon literature values in the cell systems where the drug had exerted an influence on a biochemical process at at least one half the concentration used here. While only one concentration of each potential antagonist was employed per route of administration, the dose levels were higher than those known to induce effects in other systems (10-23) and there is no reason to suspect that the eye is any less sensitive than other organs. In addition, at least 4 of the compounds were used via two routes of administration, either intravitreal and/or topical or systemic.

For all drugs, MDM #2 was given intravenously in liquid form at a dose of 5 µl/rabbit (1.93 µg total dose) which normally induces a fall in IOP of about 40% (1,2,4). Following intravitreal injections of an antagonist, MDM was administered 24 hours later; following topical application or intravenous injection of agonist or antagonist, MDM was given 30 minutes later. Twenty-four hours was deemed sufficient time for any antagonist not only to reach the target tissue (in this case, the ciliary body) but to induce and sustain a pharmacological response. Since the loss of sucrose (342 daltons) from the vitreous has a half-life of 15 hours (24), the choice of 24 hours appears reasonable for compounds of similar or larger

molecular weight, given the induction of a response in addition to the the diffusion time.

RESULTS

Group I - Adenylate Cyclase Activity

The intravenous injection of 200 to 400 µg MDM #1/rabbit in Groups I and II results in a fall in IOP of about 60% (1,2) beginning at about 4 hours and lasting for several hours. Under the assay conditions for adenylate cyclase, minimal changes were found, although variable results were obtained with NaF, and isoproterenol plus GTP, as indicated by the large standard errors (Table 2). It appears that MDM has no significant effect on ciliary epithelial adenylate cyclase nor on the responsiveness to ß-adrenergic agonists and guanyl nucleotides.

Table 2
Adenylate cyclase activities of iris-ciliary body from MDM-treated rabbits

Assay Condition	% of Control Activity
Base activity	119 ± 9.9
+ 2 x 10^{-2}M NaF	97 ± 21.4
+10^{-5}M isoproterenol	114 ± 11.6
+10^{-4}M GTP	113 ± 9.2
+ 2 x 10^{-7} GppNHp	99 ± 9.6
+isoproterenol + GppNHp	104 ± 5.5
+isoproterenol + GTP	123 ± 17.4

Values are the mean ± SEM for 4 experiments under each condition, and are given relative to parallel control, paired tissue activities. Rabbits received 200 to 400 µg MDM #1 and were assayed at 4 hours after injection.

Group II - ATPase Activity

The ATPase assays (Table 3) show that, although the Na,K-sensitive fraction is unchanged by prior *in vivo* treatment with 200 to 400 µg MDM #1 per animal, both the Mg^{2+} and ΔHCO_3^- fractions are decreased. *In vitro* treatment (5 to 12 µg per 20 mg protein) does not cause a change in any ATPase activity (except for the expected aging of the Na,K fraction; data not shown). It is apparent, therefore, that *in vivo* MDM reduces the activity of Mg^{2+} (28%) and anion-sensitive ATPase (29%) in the ciliary epithelium.

Group III - Substance P Antagonist

Substance P antagonist, although inhibiting both the irritant effects of exogenous substance P and the inflammatory response to infrared irradiation of the iris (9), had no effect on the ocular response to 100 µg MDM #1 per rabbit at concentrations far greater than those which prevent aqueous flare and miosis after exogenous substance P (9). The maximal percentage falls in IOP caused by MDM (at 360 minutes after administration) were

Table 3
Effect of MDM on ATPase of the rabbit ciliary body

	Mg^{2+}	Na^+-K^+	HCO_3^-	n
Control	13.0 ± 0.7	3.3 ± 0.2	8.4 ± 0.4	9
In Vivo Treated (6 hours after injection)	9.3 ± 0.4$^+$	3.1 ± 0.4	6.0 ± 0.4$^+$	6
In Vitro Treated (4 hours incubation)	13.6 ± 0.7	3.2 ± 0.2	7.9 ± 0.4	8

Values are the mean ± SEM of n tissues, and are in µ moles P_i liberated from ATP per hour per mg protein. +, $P < 0.001$ compared to control values. Rabbits received 200 to 400 µg MDM #1 per animal for *in vivo* determinations, and were incubated with 5 to 12 µg MDM per 20 mg protein for 4 hours.

as follows: control eye, 35.2 ± 3.9%, paired substance P antagonist-treated eye, 0.1 μM, 37.9 ± 4.4%; control eye, 45.2 ± 4.8%, paired 10 μM substance P antagonist-treated eye, 42.4 ± 5.0%. Antagonism of possible substance P activity in the eye evidently is without effect on the ocular response to MDM, despite known ocular effectiveness for 7 hours (9).

Group IV - Sugar Antagonism

Intravenous galactosamine caused a significant inhibition of the MDM-induced fall in IOP at 5, 25, and 50 μg of MDM #1, whereas glucosamine caused an inhibition at MDM doses of 5 and 50 μg. Xylose did not modify the MDM-induced response, and rhamnose inhibited the response only at 5 μg MDM (Table 4). These responses do not follow a true dose-response relationship, however, and may reflect individual rabbit differences in response to MDM, with some responding more than others.

Group V - Antagonists and Agonists

MDM (1.93 μg) was always given intravenously against various antagonists given either topically, intravitreally, or intravenously (Table 5). In no case was a statistically significant difference found between drug + MDM and MDM alone which would suggest an effect of any antagonist on the normal progression of the ocular response to MDM.

DISCUSSION

Supramaximal doses of MDM were used in several experiments in order to accentuate any possible changes which might have occurred and to ensure that a maximal response would be obtained with regard to the fall in IOP (1,2). The times chosen for the biochemical analyses were selected to coincide with the maximal fall in IOP, relying

Table 4
Effect of sugars on the MDM-induced fall of rabbit IOP

MDM Dose (μg)	Time-Zero IOP (mm Hg)	None (% Change)	Time-Zero IOP (mm Hg)	Galacto-samine (% Change)	Time-Zero IOP (mm Hg)	Gluco-samine (% Change)
5	28.1 ± 1.2 (8)	27.3 ± 0.8	24.3 ± 0.8 (8)	14.9* ± 4.1	25.4 ± 0.8 (8)	17.3* ± 3.7
10	25.4 ± 1.1 (4)	24.5 ± 3.6	25.6 ± 1.8 (4)	24.2 ± 6.6	22.4 ± 0.4 (8)	24.4 ± 3.7
25	26.2 ± 1.2 (8)	49.9 ± 2.9	26.0 ± 0.8 (8)	21.7* ± 5.4	24.3 ± 0.8 (8)	41.2 ± 2.1
50	27.8 ± 0.6 (8)	60.1 ± 2.5	23.2 ± 0.7 (8)	48.9* ± 2.4	23.0 ± 0.5 (8)	44.3* ± 2.5

	Time-Zero IOP (mm Hg)	Xylose (% Change)	Time-Zero IOP (mm Hg)	Rhamnose (% Change)
5	22.8 ± 0.7 (8)	20.8 ± 3.8	24.9 ± 0.6 (8)	16.4* ± 4.3
10	25.5 ± 0.8 (8)	34.8 ± 5.6	25.1 ± 0.7 (8)	37.8 ± 3.6
25	25.0 ± 0.7 (8)	49.7 ± 4.2	24.6 ± 0.7 (8)	42.0 ± 3.2
50	---	---	---	---

The values represent the mean ± SEM of the percentage fall in IOP 6 hours after the time-zero value (given in mm Hg). *, $P < 0.05$ compared to "no sugar" values. ---, indicates not performed. MDM #1 was given intravenously at the doses indicated.

Table 5
Effects of agonists and antagonists
on MDM-induced fall in IOP in rabbits

Drug	Dose	Drug Route	Maximal Fall in IOP: MDM Alone (5 µl)	n	MDM (5 µl) + Drug	n
Theophylline	10^{-2} M	Topical	40.8 ± 3.7	24	47.7 ± 8.5	8
	10^{-2} M	Intravitreal	46.7 ± 7.2	8	38.7 ± 5.1	8
Isoproterenol	4%	Topical	47.3 ± 2.1	12	51.2 ± 5.3	4
Guanosine 5'-(β-α-imido) triphosphate	10^{-4} M	Intravitreal	43.9 ± 6.2	8	50.1 ± 7.2	8
Mannose-6-phosphate	10^{-3} M	Intravitreal	41.5 ± 5.2	8	40.5 ± 8.0	8
	10 mg	Intravenous	-		37.1 ± 3.7	8
Sialic acid	10^{-4} M	Intravitreal	39.8 ± 4.5	7	49.9 ± 3.9	7
Tunicamycin	2 µg/ml	Intravitreal	49.2 ± 1.5	4	57.4 ± 2.4	4
Actinomycin D	10^{-5} M	Intravitreal	35.5 ± 5.2	8	50.2 ± 2.9	7
SITS	1.1 mg	Intravitreal	45.3 ± 8.5	4	32.8 ± 8.5	4
			29.9 ± 4.0	4	41.4 ± 3.5	4
	83 mg	Intravenous	38.2 ± 4.7	4	34.6 ± 4.9	4
Yeast mannan	10 mg	Intravenous	-		50.9 ± 3.1	8
	10 µg	Intravitreal	43.9 ± 1.7	8	55.1 ± 3.2	4

The maximal fall in IOP is given as the % change from an initial base-line IOP which is an average of a -30 and 0 time IOP, and is given as the mean ± SEM of n eyes. The time of the maximal fall was 5 hours after 5 µl (1.93 µg) of MDM #2 injection unless otherwise indicated.

upon the relationship between this event and any biochemical changes to be temporally related. The absence of any effects could possibly be the result of biochemical events being concluded and returned to normal levels following the initiation of the IOP changes, but this is considered unlikely in view of the slow induction of the fall in IOP and its longevity.

These studies indicate the absence of effects of MDM on adenylate cyclase of the iris-ciliary body, or on possible substance P activity in the eye. MDM significantly reduced the Mg^{2+} ATPase, and anion-sensitive ATPase, of the iris-ciliary body, but had no effect on the Na,K-stimulated ATPase. While the bicarbonate-sensitive ATPase could possibly play a role in the secretion of bicarbonate ions, a role of the Mg^{2+} ATPase in aqueous humor formation is at present unknown.

Previous studies (25) with intravitreal ouabain (0.5 µg) have indicated that a fall of 78% in aqueous humor inflow was associated with a 70% decrease in Na,K-ATPase activity (almost a 1:1 relationship) and no change in Mg^{2+} ATPase activity. It is possible that inhibition of certain ATPases can interfere with aqueous inflow, but two factors must be considered. First, the role of the Mg^{2+} and anion-sensitive ATPases are not known at present; this does not preclude their involvement in aqueous humor inflow, but the demonstrated link between Na,K-ATPase and aqueous inflow (25) argues against a direct involvement for the other ATPases. Second, if the reduction in activity

corresponds directly to a fall in aqueous inflow, as shown previously (25), then only a 30% fall in aqueous inflow would be predicted based upon the level of enzyme inhibition. Thus, unless there is considerable biochemical amplification associated with the inhibition of the Mg^{2+} ATPase or the anion-sensitive ATPase, it is difficult to reconcile the measured changes with the large (> 70%) inhibition of aqueous flow (1,2). It appears unlikely, therefore, that the inhibition of Mg^{2+} and anion-sensitive ATPases found here is related to the fall in aqueous humor inflow (1,2).

The absence of a response of adenyl cyclase to MDM differs from the response induced by cholera toxin (26); intravitreal and intraarterial injections of microgram quantities of the latter compound induced both a 50% fall in aqueous humor inflow and a stimulation of adenylate cyclase by at least two-fold. With cholera toxin, the two events occurred simultaneously over many hours. The effects of MDM and cholera toxin, both of which cause large reductions in IOP, appear to have a different biochemical basis.

Previous studies (1) have shown that prostaglandins are not involved in mediation of the response to MDM, and it appears that substance P is similarly excluded from participation in the MDM response as judged by the magnitude of the MDM response under the influence of the substance P antagonist. If MDM caused the release of substance P, it could further induce an increase in aqueous protein and a related inflammatory situation; the present data indicate that this is unlikely.

None of any of the other drugs tested, at least at the doses employed herein, caused any change in the MDM-induced response (Table 5). The concentration of all potential agonists or antagonists was higher than that needed in other systems to induce substantial effects (10-23) and the eye should, a priori, act in a similar manner to other organs towards the compound if any response were to be initiated. Several compounds were given at different doses via different routes, and these results substantiate the absence of any effect on the ocular exposure to MDM. That none of the protein or glycoprotein synthesis inhibitor compounds had any effect may reflect a potentially very slow turnover of epithelial cells in the ciliary epithelium. Preliminary studies using ^{3}H-thymidine indicate no (or at best very few) mitotic figures in either layer of the ciliary epithelium when tissues are examined after 1, 3, or 5 days after thymidine injection or between 7 days and 74 days of thymidine in the drinking water (McDonald, Elijah, and Green, unpublished data).

The partial inhibition of the MDM effect caused by intravenous galactosamine and glucosamine extend previous observations (2) where galactose, glucose, and mannose also caused some reduction of the MDM-response at 25 µg MDM. MDM has been found to contain 32 mole percent rhamnose of total sugar, 11 mole percent of xylose, 5 mole percent glucosamine, 16 mole percent glucose and 24 mole percent galactose (4). Thus, it may be that galactose and glucose, although not being the major sugars by analysis, may be important sugar residues in eliciting the ocular response to MDM.

The present data suggest that MDM might be acting to reduce IOP and aqueous humor inflow by a mechanism related to interaction with the terminal sugar moieties of the glycoproteins in the ciliary epithelium or by some modification of intracellular component by a cell-membrane permeable metabolite. Structural or chemical modification of surface proteins or intracellular events could induce profound effects on aqueous humor inflow. This perhaps suggests that other mechanisms exist for potential manipulation of intraocular pressure. Based upon the result that MDM acts only when given intravitreally in the monkey (2), we suggest that MDM acts at the basal surface of the non-pigmented epithelium. The identification of MDM may have revealed a unique means of affecting aqueous humor inflow.

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FINAL PROGRESS REPORT
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ANTIGLAUCOMA COMPOUNDS FROM CANNABIS SATIVA

The objective for this research as outlined in the initial grant proposal in 1980 stated that we would (1) isolate and characterize compounds from Cannabis that were active in lowering IOP, (2) synthesize and screen new compounds of similar structural type, (3) synthesize and screen water-soluble cannabinoids, and (4) select and screen other plants as possible sources of active compounds. As we succeeded in partially purifying and characterizing the active compounds isolated from Cannabis, the discovery of the high molecular weight of these compounds precluded our initial plan to synthesize analogues. Because these compounds contained no similarity to cannabinoids and since no water-soluble cannabinoid has been found effective in treating glaucoma, we refocussed our efforts on characterizing the very potent high molecular weight material and recently on fragmenting it in such a way that active smaller molecules could be obtained. We have continued to select and screen other plants as well. A summary of the progress made toward these objectives is given below.

A water-soluble material has been isolated from Cannabis sativa which is highly active in lowering intraocular pressure (IOP) in rabbits. MDM (marijuana-derived-material) has been purified ~200 times from crude extract to a ~500,000 dalton polysaccharide material, rich in rhamnose, galactose and galacturonic acid, containing protein rich in polar amino acids. Testing of this material for IOP-lowering effects is being carried out by Professor Keith Green of the Medical College of Georgia in collaboration with us. Professor Green's independently-supported efforts (EY04572) are dedicated to the physiological and pharmacological effects of these and other materials. MDM lowers IOP in rabbits, after intravenous injection, to 60% of maximum fall in IOP at 0.7 µg/animal and to 100% at 3 µg/animal. Injection of 0.01 to 0.001 µg, intravitreally, results in a similar fall, but the onset is delayed 12-15 hrs and the duration of action is even longer. This material is more effective and has a considerably longer lasting effect in lowering IOP than cannabinoids. It does not have the undesirable side effects of either cannabinoids or other antiglaucoma medications. Several lines of evidence suggest the activity is due to a smaller portion resulting from in vivo processing. The active portion of MDM might act as a hormone or neurotransmitter. Its mode of action is very different from that of most known effector molecules. We propose to cleave MDM either enzymically or chemically into fragment(s) without destroying activity. The resulting fragments will be characterized as to content of protein, sugar, uronic acids and amino sugars; molecular weight; electrophoretic mobility; and IOP-lowering activity expressed as ED_{40}. ED_{40} is the estimated dose (in µg) giving a 40% lowering of IOP (60% of maximum). This material has potential as a topically active glaucoma medication without psychoactive effects. Further study of the physiological effects of the active portion of MDM could lead to elucidation of the mode of action of an unknown class of receptors.

We have surveyed a number of other plants. One of them, Burley tobacco, yielded a crude extract which is as active as that of Cannabis, but its chemical composition is much different. If the differences in composition are related to IOP-lowering activity, isolation of the active portion from Burley might be easier than from Cannabis.

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